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Award Number: W81XWH-13-2-0036

TITLE: Preclinical and Clinical Development of Low Dose Methamphetamine for the Treatment of Traumatic Brain Injury

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REPORT DATE: April 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2014	Annual	1 April 2013- 31 March 2014

4. TITLE AND SUBTITLE
Preclinical and Clinical Development of Low Dose Methamphetamine For the Treatment of Traumatic Brain Injury

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-2-0036

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Fort Detrick, Maryland 21702-5012

U.S. Army Medical Research and Materiel Command

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This proposal addresses additional preclinical characterization of low dose methamphetamine as a neuroprotective agent for TBI. We have previously demonstrated that treatment with methamphetamine within 12 hours after TBI significantly improved cognition and functional behavior in 2-3 month old male Wistar rats. These studies were intended to examine the therapeutic effects of methamphetamine in female and aged male rats. We also repeated a MRI time course study with the intent of determining if there was a dose response effect associated with methamphetamine induced white matter track remodeling. We have determined that female rats represent a poor model for TBI. Unlike humans, female rats have a 3-day estrous cycle. This means they are endogenously exposed to the neuroprotective effects of estrogen and progesterone before, during and after injury. This allows them to recover to near normal levels within a few weeks of after injury. In this context, it is not possible to observe any kind of drug effect on cognitive or behavioral outcomes. We have determined that methamphetamine does not exhibit a therapeutic effect in aged rats. A number of changes exist in aged rats that could account for this observation. First, they have reduced dopamine receptors. We have shown that methamphetamine-mediated neuroprotection is dependent, in part, on the activation of dopamine receptors. This reduction of dopamine receptors also contributes to a condition of chronic inflammation. We have shown that methamphetamine reduces neuroinlammatory signaling. Thus, the reduction of dopamine receptor mediated signaling could explain the loss of protective effect in aged rats. The MRI time course series confirmed our previous results but we did not observe a dose response effect. However, we did determine that methamphetamine treatment significantly increased neurovascular staining in the perilesional region. This diata suggests that methamphetamine treatment also enhances angiogenesis after injury.

15. SUBJECT TERMS
none provided

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
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INTRODUCTION

We have previously demonstrated that treatment with low dose methamphetamine results in significant improvements in cognition, functional behavior (Rau et al., 2012, 2014). We have also demonstrated that methamphetamine treatment significantly preserved axons, dendrites and enhanced white matter track remodeling in the perilesional region following severe TBI (Ding et al., 2013). Finally, we recently demonstrated that methamphetamine treatment significantly reduces seizure susceptibility following severe TBI. The purposes of the currant studies are: 1) to investigate the potential therapeutic effects of methamphetamine on female and aged male rats; 2) to investigate the dose response effects of methamphetamine on white matter track remodeling; and 3) to submit an amended IND application to FDA in preparation for a phase IIA dose escalation safety study. The aged and female studies were proposed in an effort to be compliant with recent recommendations

KEY WORDS

Traumatic brain injury, neuroprotection, methamphetamine, aged rats, female rats, MRI, neurological severity score, foot fault, Morris water maze, confidential investigator brochure, investigational new drug application.

OVERALL PROJECT SUMMARY

Aim 1: Test the hypothesis that low dose methamphetamine is neuroprotective when administered to aged or female rats after severe TBI.

We used the lateral fluid percussion injury model to induce severe TBI in 10-12 month old male Wistar rats. Rats were treated with a bolus IV injection of 0.4 mg/kg methamphetamine followed by continuous IV infusion with 0.5mg/kg/hr methamphetamine for 24 hours. Control rats received the same injection and infusion but with saline. Only rats with a severe TBI (neurological severity score of 10 or higher) were used. Functional behavior was assessed in all rats based on neurological severity scores (NSS) and foot fault assessments on day-1 post injury and then again weekly for 5 weeks. As figure 1 indicates, there was a significant difference in both the NSS and foot fault outcomes between uninjured sham rats and TBI injured rats. However, unlike the young rats, methamphetamine did not induce a significant improvement in for behavioral outcomes (NSS or foot fault) in the aged rats over the 5-week test period. These data suggest that methamphetamine is not neuroprotective in aged Wistar rats.

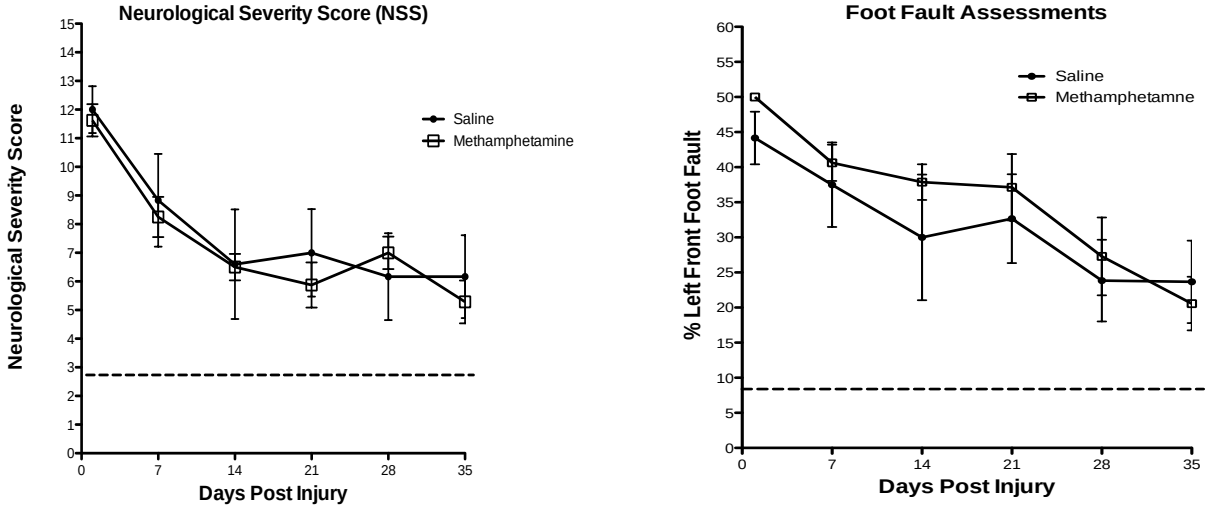


Figure 1. Methamphetamine does not improve behavioral outcomes in aged rats after severe TBI. Unlike younger rats (2-3 months old), there were no significant differences observed between saline and methamphetamine treated aged rats with either of the behavioral outcome measures tested. The dashed line indicates the average score observed for uninjured, sham-operated control rats. The significant difference between sham controls and TBI rats indicates an injury effect and that NSS and foot fault are appropriate outcome measures.

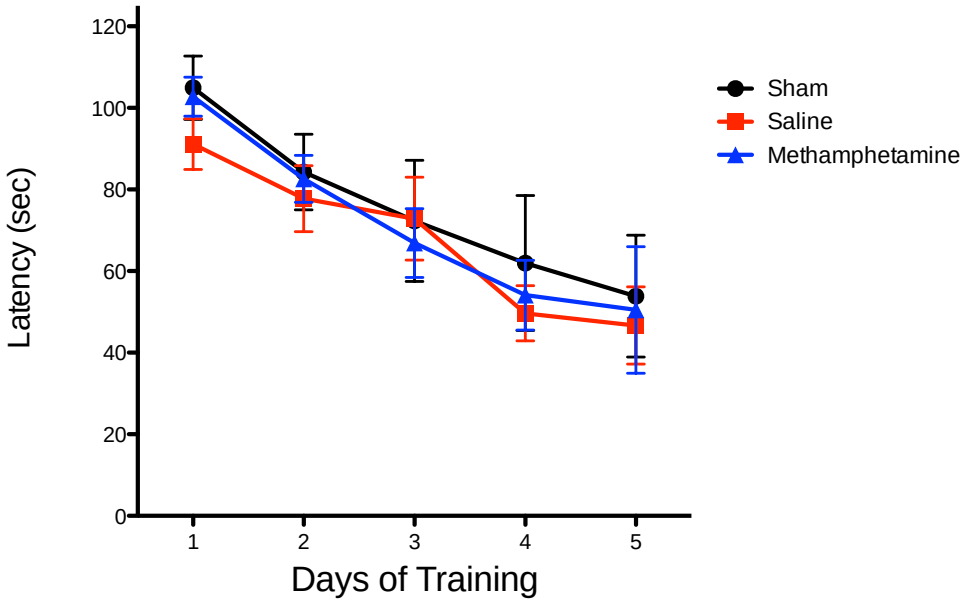


Figure 2. No significant differences in learning ability were observed between sham, saline or methamphetamine treated rats in the Morris water maze. Latency (time to locate submerged escape platform) is shown for sham (black), saline (red) and methamphetamine (blue) treated rats.

We further tested the potential of methamphetamine to improve cognition in aged rats similar to what we saw in younger rats (Rau et al., 2012, 2014). At 6 weeks post injury/treatment all rats were trained for 5 days in the Morris water maze. Each rats received four training sessions/day. The time each rat took to learn the location of a submerged escape platform (latency) was recorded. Figure 2 shows the average latency times for rats in each treatment group on each of the five training days. Even the uninjured, aged rats showed significant learning impairment relative to latency times we previously observed for young rats. Due to the poor performance of the aged sham controls in the Morris water maze, it was not possible to detect a difference due to injury and therefore impossible to detect a difference due to a drug effect.

As mentioned above, methamphetamine mediates neuroprotection in part, through the activation of dopamine receptors. Its been shown that aged rats and humans have reduced expression of dopamine receptors. Therefore, we reasoned that increasing the dose in aged rats might be required to see a therapeutic effect. The first group of aged rats received a bolus injection plus IV infusion with 0.5 mg/kg/hr for 24 hours. Therefore, we tested a dose of 1.0 mg/kg/hr in a second cohort. Five aged, male, Wistar rats were treated at this higher dose. All five rats died within the 24-hour dosing period. Given this high rate of mortality we did not feel that it was ethical or of scientific value to continue and include additional animals. Given this observation, we reevaluated the doses administered and compared the absolute dose given versus the dose/weight administered to young and aged rats. This seemed relevant due to the fact that the aged rats were substantially heavier (average weight = 576 $\pm$ 59g) compared to the younger rats (average weight = 377 $\pm$ 45g). As a consequence, the average absolute total dose of methamphetamine previously given to young rats was 4.68 mg. In contrast, the average absolute total dose given to the first cohort of aged rats was 7.15 mg. This represents 1.5 times the absolute dose given to younger rats. Furthermore, the second aged cohort, that experienced 100% mortality, was given an average absolute total dose of 13.85 mg. Thus, the average absolute total dose given to the second aged cohort was almost three times higher than the dose given to the young rats.

We next examined the neuroprotective potential of methamphetamine following a severe TBI in young (2-3 months old), female, Wistar rats. Again, only rats that scored 10 or higher on the NSS assessment at 24 hours after injury were included in these studies. This insured that all rats received a similar injury of sufficient severity. Behavioral assessments (NSS and foot fault) were conducted on all rats on a weekly basis for five

weeks post-injury. As Figure 3 indicates, there were no significant differences in either NSS or foot fault assessment scores during the testing period.

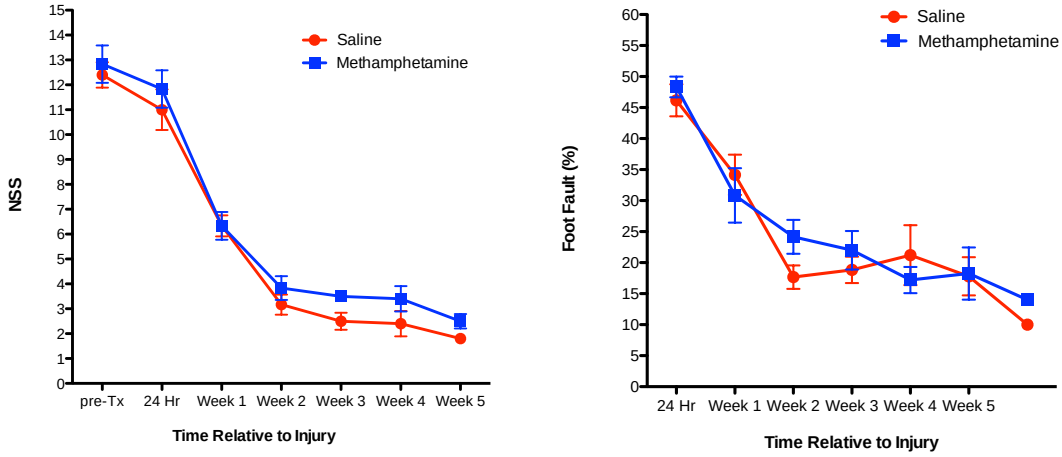


Figure 3. Methamphetamine does not induce a significant improvement in female rats after severe TBI. NSS (left panel) and Foot Fault (right panel) assessments over a 5 week time course following severe TBI are shown for saline (red) and methamphetamine (blue) treated rats.

We previously demonstrated that methamphetamine treatment significantly improved cognitive outcomes following severe TBI in young, male Wistar rats. Therefore, we wanted to further examine the potential of methamphetamine to improve cognitive function in female rats. Interestingly, unlike the behavioral assessments, saline treated TBI control rats did exhibit impairment in the Morris water maze one month after injury (Figure 4). Unfortunately, although the data suggested that methamphetamine treatment caused a trend toward improvement, a significant difference from saline treated controls was not observed.

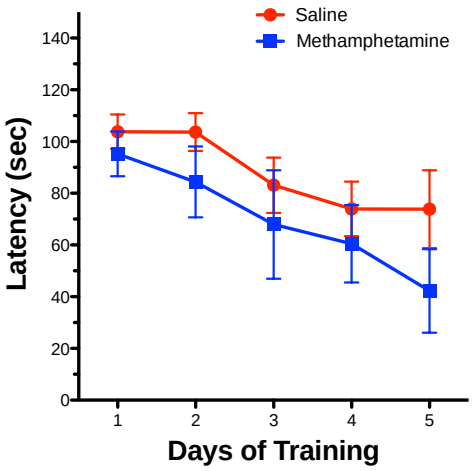


Figure 4. Cognitive testing of female Wistar rats after severe TBI. Female rats were tested in the Morris water maze 30 days after receiving a severe TBI. Latency (time to locate escape platform) over 5 training days is shown for rats treated with saline (red) or methamphetamine (blue) following severe TBI.

Aim 2: Test the hypothesis that low dose methamphetamine can improve neurovascular changes and axonal remodeling over time.

MRI measurements after TBI with and without Methamphetamine treatment: MRI measurements were performed one day before TBI, 1 and 3 days post TBI (using the controlled cortical impact model), and then weekly for 6 weeks. MRI studies were performed using T1, T2, T2*, and fractional anisotropy (FA). Stereotactic ear bars were used to minimize movement during the imaging procedure. During MRI measurements, anesthesia was maintained using a gas mixture of N_2O (69%), O_2 (30%) and isoflurane (1-1.5%). Rectal temperature will be maintained at 37°C using a feedback controlled water bath. After the last MRI measurements, the animals were sacrificed. White matter changes in the brain tissue were assessed by specific immuno-histochemical staining. The analyzed data were obtained from ten control and eight treated animals.

T_2 measurement: T_2 was measured using a Carr-Purcell-Meiboom-Gill multislice multiecho (six echo) MRI. A series of four sets of images (13 slices for each set) were obtained using TEs of 15, 30, 45, 60, 75, and 90 msec and a TR of 4.5 sec. Images were produced using a 32 mm FOV, 1 mm slice thickness, 128 x 128 image matrix.

T_2^* measurement: T_2^* was obtained using a gradient echo multislice multiecho sequence. A TR value of 4.5 sec was used with echo times of 3.5, 15, 20, 25, 30, and 35 msec. Images were produced with a 32mm FOV, 1mm slice thickness, 13 slices, and 128 X 128 image matrix.

***Diffusion Tensor MRI (DTI) measurement*:** DTI was acquired using pulsed gradient spin-echo echo-planar (EPI) sequence with echo-planar readout. The field of view was 32 mm; two average, 128x128 imaging matrix, 1 mm slice thickness with 13 slices, TR = 800 ms and TE = 36 ms, 16 shots, 4 averages, 15 diffusion attenuate weighted images with b = 1200, 600, and 0 s/mm^2 in each slice.

***MRI ANALYSIS*:** The TBI lesion was identified on the MRI T_2 map. The lesion area on each slice of T_2 map was specified by those pixels with a T_2 value higher than the mean plus twice the standard deviation (mean + 2SD) measurements provided by the normal tissue on the pre-TBI T_2 map. Lesion volume was obtained by adding all the areas measured on individual slices and multiplying each area by the slice thickness.

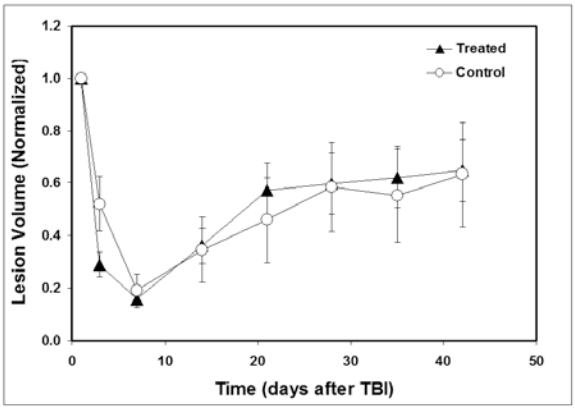
The volume of the lateral ventricle was measured on T_2 maps at a fixed structural location presented by six contiguous coronal T_2 slices using similar criterion as described above to identify the ventricular area on each slice. The ventricular volume was obtained by adding all the areas measured on individual slices and multiplying each area by the slice thickness. The ventricle volumes measured at various time points were normalized for each animal to the ventricular volumes measured at pre-TBI.

MRI regions of interest (ROI) were identified in the TBI core and recovery areas. Two ROIs were selected for analysis of MRI parameters. The first ROI, the TBI core area, was identified by using the threshold T_2 value of mean + two standard deviations from the T_2 value measured in the pre-TBI T_2 maps obtained 6 weeks after stroke. The second ROI, the TBI recovery area, was identified by subtracting the TBI core areas obtained 6 weeks after stroke from the TBI area in T_2 maps obtained 24 hours after TBI.

MRI Results:

The TBI lesion volumes were measured using T_2 maps at 24 hours, and weekly from 1 to 6 weeks post-TBI and normalized to the lesion volume obtained 24 hours after TBI. There was a marginal transient reduction of normalized lesion volume at 3 days (p=0.055) after TBI. However, there was no treatment effect on the normalized TBI lesion volume compared to controls with the analyzed data (Figure 5).

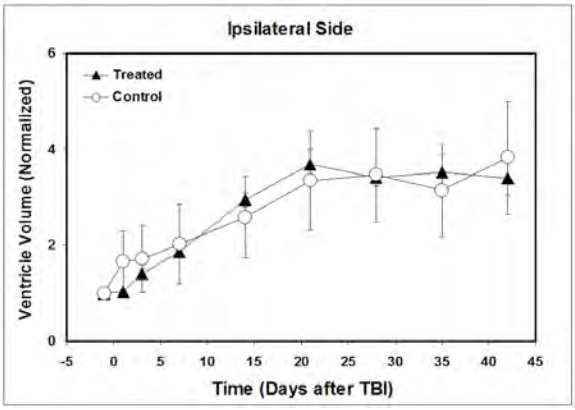
Figure 5



The relative ventricular volumes were measured using T_2 maps before TBI, and at 24 hours, and weekly from 1 to 6 weeks post-TBI and normalized to the ventricular volume obtained before TBI. There was no treatment effect on normalized ventricular volume compared to controls (Figure 6).

Measurements of T_2 and T_2^* were performed on the TBI core and recovery ROIs (perilesional region). The relative changes of MRI measurements in the TBI core ROI (TBI core/Pre-TBI core) and recovery ROI (TBI recovery/Pre-TBI recovery) were calculated and used in the analysis. There was no treatment effect on the TBI relative T_2 and T_2^*, compared to controls with the analyzed data.

Figure 6



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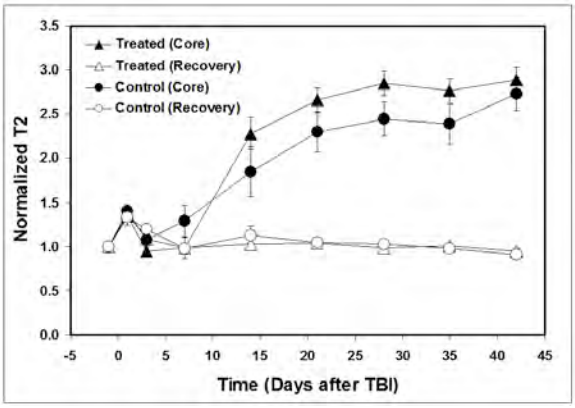

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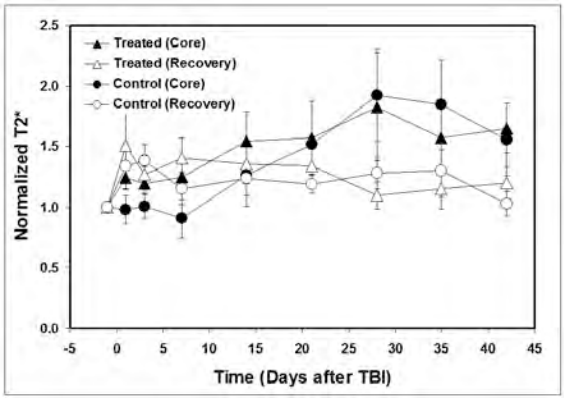


Figure 7. T2 (A) and T2* (B) image analysis of the injury core (filled symbols) and perilesional region (open symbols) for saline treated (circles) and methamphetamine treated (triangles) rats. Time course shown is days after TBI.

Measurements of FA were performed on the TBI core and recovery ROIs to determine white matter track remodeling. The relative changes of FA ($FA_{post\_TBI}/FA_{pre\_TBI}$) in the TBI injury core and recovery ROI (perilesional region) were calculated and used in the analysis. The methamphetamine treated group revealed large increases in FA in the TBI recovery regions compared with the saline treated control group, and significant differences in FA were detected at 5 (p=0.020) and 6 (p=0.017) weeks after TBI (Figure 8). There were no significant differences in FA in the TBI core regions between treated and control groups.

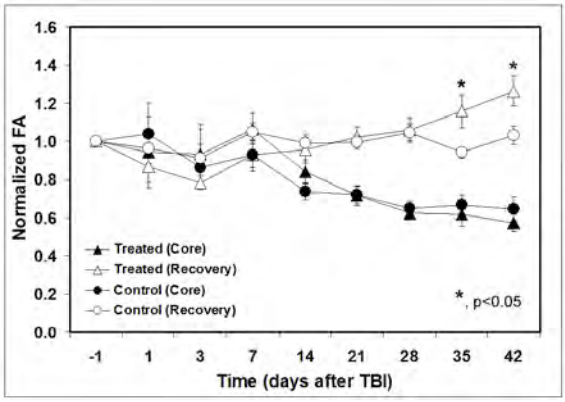


Figure 8. T2 (A) and T2* (B) image analysis of the injury core (filled symbols) and perilesional region (open symbols) for saline treated (circles) and methamphetamine treated (triangles) rats. Time course shown is days after TBI.

***Immunohistochemistry staining*:** At 42 days after TBI, rats were anesthetized intraperitoneally with chloral hydrate, and perfused transcardially with saline, followed by 4% paraformaldehyde. Brains were isolated, post-fixed in 4% paraformaldehyde for 2 days at room temperature, and then processed for paraffin sectioning. Using light microscopy and laser scanning confocal microscopy (LSCM), we measured a composite index of independent measurements of axon, microvessel, dendrite, synapse immuno-histochemistry (Bielshowsky /fast blue, EBA, SMI-310, SMI-32, MAP-2 and synaptophysin) and lesion volume.

Neurobehavioral functional tests: Neurological function was monitored by modified neurological severity scores (mNSS) and foot-fault test. Baseline neurological function was established for all animals prior to TBI and again on days 1, 7, 14, 21, 28, 35, and 42-post injury. On day 38 to day 42, the Morris Water Maze (MWM) test was performed to assess the impact of methamphetamine on cognitive function (learning and memory) following TBI.

Histopathology (CCI model):

Bielschowsky and Luxol fast blue labeled axons and myelin, respectively, and were used to monitor changes in cerebral white matter in the TBI boundary ROI (perilesional region). Significant increases ($p<0.034$) in Bielschowsky and Luxol Fast Blue staining area in the TBI boundary ROI were detected in the methamphetamine treated group (35.5±19.8) compared with the control group (17.6±3.0) (Figure 9). Likewise, a significant increase ($p<0.001$) in the number of blood vessels per mm^2 as monitored by EBA immunoreactivity was observed within the perilesional region in the methamphetamine treated group (422.4±31.5) compared to the saline treated control group (323.8±49.7) (Figure 10). In addition, significant increases were observed in dendrites labeled with MAP2 ($p<0.001$) (Figure 11), axons labeled with SMI-32 ($p=0.002$) (Figure 12), and synapses labeled with synaptophysin ($p<0.001$) (Figure 13) within the perilesional region of methamphetamine treated rats compared to the saline treated control group.

Figure 9

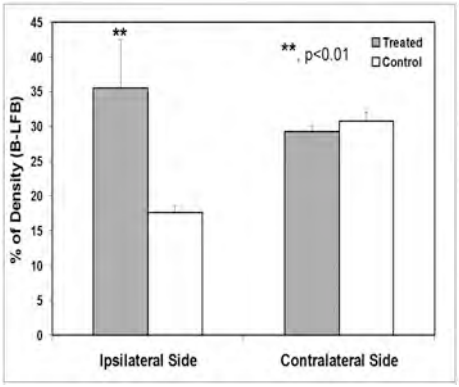


Figure 10

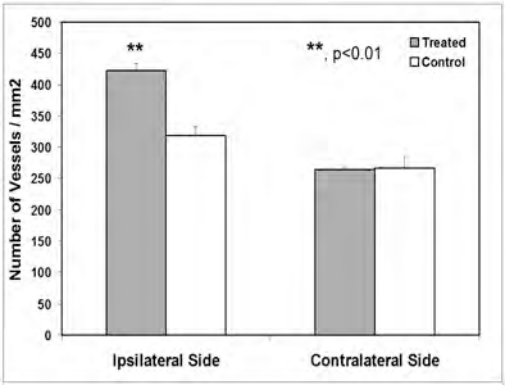


Figure 11

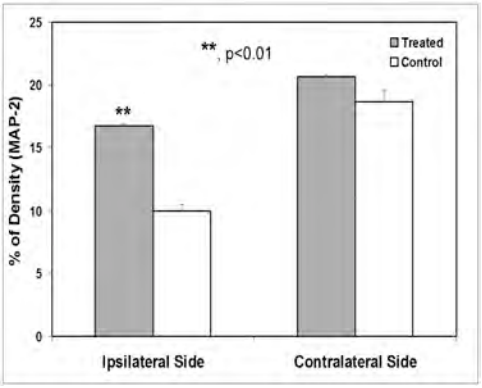


Figure 12

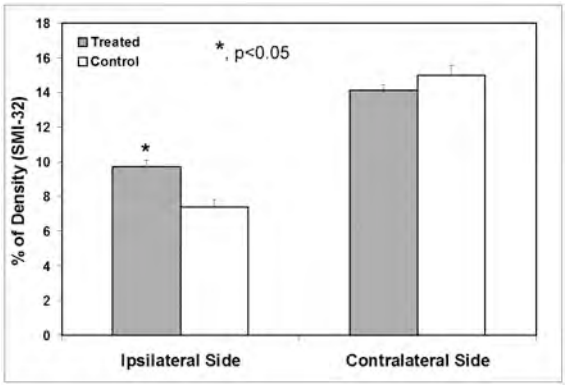
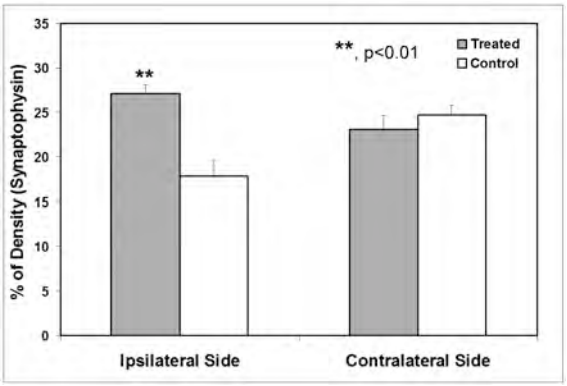


Figure 13



Functional behavior (CCI model)
Significant improvements in functional behavior were detected in the Methamphetamine treated group compared to the control group. Methamphetamine treated rats had significantly lower Neurological severity scores (mNSS, 1 day to 6w, $p<0.05$) and foot-fault (7 days to 4w, and 6 w $p<0.05$) tests (Figure 14).

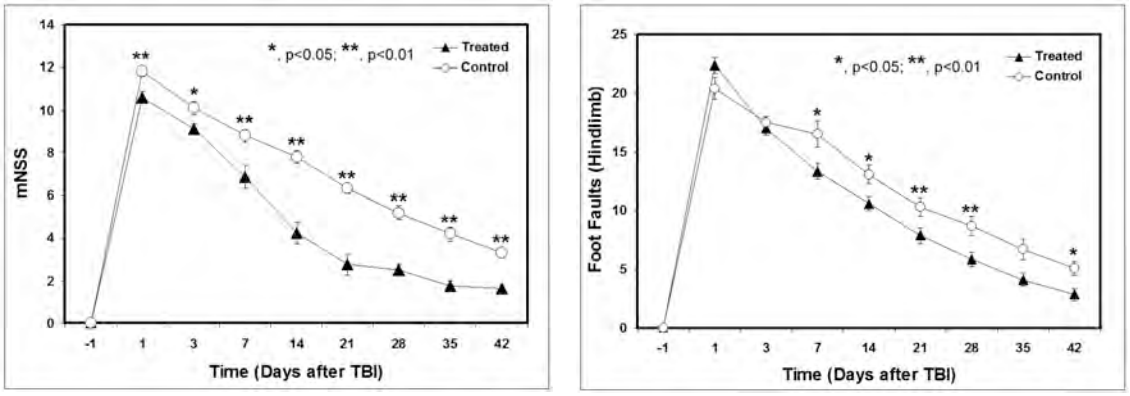


Figure 14. Methamphetamine increases functional behavior following severe TBI in CCI model. Serial mNSS (left panel) and foot fault assessments (right panel) are shown for methamphetamine treated (dark triangles) and saline treated controls (open circles).

In summary, low dose (a bolus injection of 0.4mg/kg followed by an infusion of 0.5mg/kg/hr for 24 hrs) methamphetamine treatment starting at 8 hours after TBI in rats significantly improved the recovery of behavioral functions in rats by lowering mNSS and foot-fault scores after TBI, compared with saline treatment. The treatment promoted neurological recovery after TBI, which was detected by MRI measurement of FA and confirmed by immunohistochemical outcomes. The increased FA, indicating a more reorganized white matter, may contribute to the functional recovery after TBI in rats.

Aim 3: Secure FDA approval of a phase I/II clinical trial plan and obtain an amended IND in preparation for initiating human clinical testing in TBI patients.

We have received correspondence from Dr. Russell Katz, M.D., (Director of the Division of Neurology Products, CDER), in which the FDA has requested toxicity studies in rats and dogs be completed before an amended IND and additional clinical trials can be approved. This request is consistent with the FDAs guidance for industry regarding nonclinical evaluation of reformulated drug products intended for administration by a route that is different from the currently approved formulation. Methamphetamine is currently FDA approved for oral administration. Therefore, the administration of an IV formulation, as we are proposing, necessitates the additional toxicity studies requested by the FDA. The FDA Guidance for Industry: *M3(R2) Non Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceutical*, also recommends that safety pharmacology studies be conducted as well. Given the potential for methamphetamine to induce abnormal cardiac rhythms at higher doses, we also feel that it is prudent to conduct a cardiovascular safety pharmacology study in dogs. The completion of the proposed preclinical toxicity and safety studies, combined with the long clinical history and safe use of methamphetamine at low doses, will provide a well defined, safe dose range for testing in a phase IIA dose escalation safety study in TBI patients. A JWMRP proposal has been submitted and we hope this support will become available to complete these studies. Without the completion of these toxicity studies it will not be possible to complete this aim.

Aim 4: Prepare a confidential investigator brochure (CIB) to support the submission of an amended IND application to the FDA.

Draft of the final FDA reports that are to be included in the CIB are currently being completed.

Key Research Accomplishments

- We have determined that methamphetamine does not induce significant improvements in functional behavior or cognition in aged rats following severe TBI in the LFP injury model.
- We have determined that NSS and foot fault assessments represent very poor outcome measures in female rats following a severe TBI in the LFP model.
- We have demonstrated that methamphetamine treatment does not produce a significant improvement in cognition in female rats following severe TBI with the LFP model.
- We have shown that methamphetamine treatment significantly improves white matter track remodeling, preserves axons, dendrites and synapses within the perilesional region following severe TBI in the CCI model.
- We have shown that methamphetamine treatment significantly improves labeling of blood vessels within the perilesional region following severe TBI in the CCI model.

Conclusions

We have demonstrated that methamphetamine mediates neuroprotection in part, through dopamine dependent activation of D1 and D2 receptor and a PI3K/AKT signaling cascade. Aged rats and humans have reduced dopamine receptors. This may account for the reduction in methamphetamine efficacy in aged rats.

Unfortunately, female rats have a three-day estrous cycle. This means that they are exposed to the neuroprotective effects of progesterone and estrogen before, during and after injury. As a consequence, negative control, female rats that were exposed to saline recovered functional behavior that was similar to that observed in uninjured rats within about 2 weeks post injury (Figure 3). Thus, female rats offer a very poor model to examine a potential drug effect following sever TBI, as it is highly unlikely that we can improve a rats functional behavior beyond normal

We previously demonstrated that treatment with an IV bolus of 0.42 mg/kg followed by IV infusion with 0.05 mg/kg/hr for 24 hours produced increased white matter track remodeling at 5 and 6 weeks post injury in the CCI TBI model. The goal of this study was to test the hypothesis that increasing the dose to a bolus injection of 0.42 mg/kg followed by continuous IV infusion with 0.5 mg/kg/hr for 24 hours would increase the therapeutic effect. Our results suggest that there is not a dose dependent effect on methamphetamine mediated white matter track remodeling. We did also observe that methamphetamine enhances blood vessel labeling in the perilessional region region. This data suggests that methamphetamine promotes angiogenesis.

Publications, Abstracts, and Presentations:

There have be no publications or presentations of this data within the past year

Inventions, Patents and Licenses

International patent application no. PCT/US2007/076034 was filed on August 15, 2007, entitled: "METHOD OF REDUCING NEURONAL CELL DAMAGE". The application is directed to and claims methods of treating or reducing the occurrence of neuronal cell damage in a subject having a transient cerebral hypoxia and/or ischemic condition (e.g., stroke, traumatic brain injury) by administering a neuroprotective amount of methamphetamine to the subject within 16 hours after onset of the condition. National phase applications were filed and are pending in the following countries: Australia; Brazil; Canada; China; Europe; Hong Kong; India; Indonesia; Israel; Japan; Mexico; Philippines; Republic of Korea; Russian Federation; South Africa; and the United States. All applications claim priority to U.S. provisional application. no. 60/839,974, filed on August 23, 2006. Claims have now been awarded in the Belgium, Switzerland, Germany, Spain, France, Great Britain, Italy, Russia, Japan, Australia, and South African. Patent claims are about to be issued in Mexico and Philippines. The patent is still being prosecuted in the US.

Reportable Outcomes

The current studies suggest that we should limit participation in future clinical trials involving methamphetamine treatment of TBI to younger patients (perhaps less than 45 years of age). It seems a bit unnecessary to exclude females from future trials based on the current rat data. However, this may be a consideration.

Other Achievements

A JWMRP application based on the advancement of the current work has been submitted.

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Appendices

Neuropharmacology 61 (2011) 677–686

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Low dose methamphetamine mediates neuroprotection through a PI3K-AKT pathway

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ARTICLE INFO

Article history:
Received 29 June 2010
Received in revised form
10 May 2011
Accepted 11 May 2011

Keywords:
Stroke
Methamphetamine
Dopamine
Neuroprotection

ABSTRACT

High doses of methamphetamine induce the excessive release of dopamine resulting in neurotoxicity. However, moderate activation of dopamine receptors can promote neuroprotection. Therefore, we used *in vitro* and *in vivo* models of stroke to test the hypothesis that low doses of methamphetamine could induce neuroprotection. We demonstrate that methamphetamine does induce a robust, dose-dependent, neuroprotective response in rat organotypic hippocampal slice cultures exposed to oxygen–glucose deprivation (OGD). A similar dose dependant neuroprotective effect was observed in rats that received an embolic middle cerebral artery occlusion (MCAO). Significant improvements in behavioral outcomes were observed in rats when methamphetamine administration delayed for up to 12 h after MCAO. Methamphetamine-mediated neuroprotection was significantly reduced in slice cultures by the addition of D1 and D2 dopamine receptor antagonist. Treatment of slice cultures with methamphetamine resulted in the dopamine-mediated activation of AKT in a PI3K dependant manner. A similar increase in phosphorylated AKT was observed in the striatum, cortex and hippocampus of methamphetamine treated rats following MCAO. Methamphetamine-mediated neuroprotection was lost in rats when PI3K activity was blocked by wortmannin. Finally, methamphetamine treatment decreased both cleaved caspase 3 levels in slice cultures following OGD and TUNEL staining within the striatum and cortex in rats following transient MCAO. These data indicate that methamphetamine can mediate neuroprotection through activation of a dopamine/PI3K/AKT-signaling pathway.

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1. Introduction

Methamphetamine induces the extracellular accumulation of dopamine at nerve terminals by modulating the activity of dopamine transporters (DAT) and vesicular monoamine transporters (VMAT) (Kita et al., 2009; Krasnova and Cadet, 2009). Exposure to high and repetitive doses of methamphetamine has been reported to induce excessive dopamine release within the synaptic cleft, leading to toxic levels of aldehydes and quinones (Cadet et al., 2007). In addition, dopamine can increase the production of hydrogen peroxide and nitric oxide resulting in the generation of the reactive nitrogen species (RNS) peroxynitrite (Cadet and Krasnova, 2009). High doses of methamphetamine are also linked to excessive glutamate release in the striatum and hippocampus resulting in excitotoxicity (Nash and Yamamoto, 1993). Increased extracellular glutamate levels also cause the production of reactive nitrogen species and activation of calcium-dependent proteases and cytoskeletal damage (Thrash et al., 2009). High doses of methamphetamine alter energy metabolism resulting in decreased succinate dehydrogenase activity leading to mitochondrial dysfunction (Quinton and Yamamoto, 2006). Thus, the combination of reactive oxygen species, reactive nitrogen species, excitotoxicity, and mitochondrial dysfunction are linked to methamphetamine-induced loss of dopaminergic nerve terminals throughout the ventral tegmental area, substantia nigra, hippocampus, prefrontal cortex and cortex (Hanson et al., 1998).

In contrast, it has been suggested that activation of dopamine receptors may elicit a neuroprotective response. For example, D1 dopamine receptors (D1R) may interact directly with NMDA receptor subunits (Lee et al., 2002). As a consequence, data suggest that activation of D1R may reduce NMDA receptor mediated Ca^{2+} currents in hippocampal neurons and decrease excitotoxicity in

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0028-3908/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2011.05.010

a phosphoinositol-3 kinase (PI3K) dependent manner (Lee et al., 2002). D2 dopamine receptors (D2R) may modulate AMPA receptor cell surface expression through indirect interactions with the GluR2 subunit via N-ethylmaleimide sensitive factor (Zou et al., 2005). D2R activation also elicits an AMPA dependent increase in PI3K activation. In addition, D2R activation protects rat cortical neurons from glutamate excitotoxicity by activating anti-apoptotic signaling through AKT and up-regulating Bcl-2 expression (Kihara et al., 2002).

To date, no neuroprotective agents have proven efficacious in humans following stroke. However, the use of amphetamines in combination with task specific training in the rehabilitation phase following stroke has shown potential for improved recovery of motor skills (see Martinsson and Eksborg, 2004). It has been suggested that under these conditions, amphetamine may facilitate improved recovery by influencing brain plasticity (Gold et al., 1984). Although treatment with amphetamine during rehabilitation may improve function, it has not been investigated as an acute neuroprotective agent following stroke. In fact, the repetitive administration of high dose methamphetamine (4 doses of 10 mg/kg IP every 2 h) before stroke actually potentiates ischemia-induced damage in the cortex and striatum (Wang et al., 2001). However, the potential effects of acute low dose methamphetamine administration after stroke have not been investigated. Therefore, the objective of this proof-of-concept study was to use *in vitro* and *in vivo* models of stroke to test the hypothesis that low dose methamphetamine produces a neuroprotective response when applied as an acute treatment following injury. We present evidence indicating that methamphetamine generates a robust neuroprotective response that is dependent, in part, on dopamine-mediated activation of the PI3K-AKT signaling pathway.

2. Materials and methods

2.1. Hippocampal slice cultures and oxygen–glucose deprivation

All experimental animal procedures were approved by the University of Montana Institutional Animal Care and Use Committee in accordance with National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023). Hippocampal slice cultures were prepared from the brains of 7-day-old Sprague-Dawley rat pups as previously described (Selkirk et al., 2005). After 7 days in culture, slices were exposed to oxygen–glucose deprivation. A glucose free balanced salt solution (BSS) composed of 120 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 20 mM HEPES, 25 mM sucrose; pH 7.3 was bubbled for 1 h with 5% CO_2/95% N_2 at 10 L/h. Slices were washed 6 times in deoxygenated SBSS to remove residual glucose, transferred into deoxygenated SBSS and placed in a 37 °C chamber (Pro-Ox) with an oxygen feedback sensor that maintained gas levels at 0.1% O_2, 5% CO_2, 94.4% Nitrogen for 60 min. After OGD, the slices were immediately transferred back into pre-warmed Neurobasal media (containing B27 without anti-oxidants) under normal oxygen conditions. Slices treated with methamphetamine in the dose-response study were placed in pre-warmed Neurobasal media containing 1 μM–8 mM methamphetamine immediately after OGD. For the time course studies, 100 μM methamphetamine was added at 2, 4, 8, or 16 h after OGD. Neuronal damage was determined by staining slices with propidium iodide (PI; Molecular Probes, Eugene, OR) and quantifying the relative fluorescence intensity (excitation 540/emission 630) using ImagePro Plus software (Media Cybernetics, Silver Springs, MD). PI was added to the media at a concentration of 2 μM (Noraberg et al., 1999) 4 h prior to OGD. Images were taken of the hippocampal slices prior to OGD to establish baseline fluorescence. After OGD, slices were placed in normal media containing 2 μM PI and imaged again at 24 h post-OGD using fluorescence optics with an Olympus IMT-2 microscope and a Hamamatsu camera. The total fluorescent intensity in each slice was determined using ImagePro Plus software (Media Cybernetics, Silver Springs, MD) and all values were expressed as the percent change from untreated slices exposed to OGD.

2.2. Western blot analysis

Rat hippocampal slices were harvested from inserts and pooled (4–5 slices per insert) in 200 μl of SDS lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% triton x-100 and Phosstop Phosphatase inhibitor cocktail from Roche and Protease Inhibitor Cocktail Set III, EDTA-free). Tissue was ground for 30 s, sonicated for 5 s on ice, and centrifuged at 14,000 × *g* at 4 °C for 10 min. Protein content was determined by Bradford assay and 30–50 μg of total protein was prepared with Lamelli sample buffer and loaded onto NuSep Long Life 10 gradient (4–20%) gels. The gels were transferred to PVDF membrane (BioRad, Immun-Blot; 0.2 μM pore size) for 60 min at 100 V on ice, blocked in 5% non-fat dry milk/TBST for 1 h, and incubated overnight 4 °C with primary antibody (Cell Signaling; AKT 1:1000, pAKT 1:1000) in 5% non-fat milk. Blots were incubated with donkey anti-rabbit secondary antibody (1:20,000 for AKT; 1:2000 for pAKT, Thermo Scientific) in 5% BSA for 1 h and then washed 3 times for 5 min in TBST. Washed blots were then developed with an Amersham ECL Plus kit (GE, Piscataway, NJ) and imaged using the BioRad Chemidoc system. Densitometry was performed using Quantity One software. Blots were stripped using Restore Western Blot Stripping buffer (Thermo Scientific, Pierce, Rockford IL), washed three times in TBST, and blocked for 1 h in 5% non-fat dry milk and TBST. Blots were incubated overnight at 4° C with a monoclonal antibody for b-actin (Sigma) at 1:45,000 and developed with Amersham ECL Plus (GE, Piscataway, NJ). All samples were normalized to b-actin as a loading control prior to statistical analysis. For cleaved caspase 3 analysis, rat hippocampal slices were homogenized in cell lyses buffer (50 mM Tris–HCL, 150 mM NaCl, 1% triton x-100 and Phosstop Phosphatase inhibitor cocktail from Roche and Protease Inhibitor Cocktail Set III, EDTA-free) on ice. Samples were incubated on ice for an hour with intermittent vortexing to maximize cell lyses. Samples were then spun at 10,000 × *g* for 20 min at 4 °C to obtain total soluble protein. Total soluble protein concentrations were determined with the Bradford assay. Aliquots were stored at −80 °C. 50 μg of total protein were run on a 15% Tris–HCl SDS–PAGE gel (BioRad). The gels were then transferred to PVDF membranes that were blocked with normal in a solution of 5% NFM/TBS-T (0.1%) overnight at 4 °C. Rabbit anti-Cleaved Caspase-3 antibody (Cell Signaling, #9661) was added at a dilution of 1:1000 and incubated overnight at 4 °C. Secondary anti-rabbit antibody (Vector) at 1:2000 was incubated at room temperature for 1 h. Membranes were imaged with ECL Plus (GE Healthcare/Amersham) on a Chemidoc XRS densitometer (BioRad). After imaging, membranes were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) and probed with anti-b actin. The density of all protein bands was analyzed using Quantity One software (BioRad).

2.3. Neurotransmitter analysis by RP-HPLC

Levels of dopamine and homovanillic acid (HVA) in rat organotypic hippocampal slice cultures (RHSC) was measured using reverse-phase high performance liquid chromatography (RP-HPLC) with electrochemical detection, as previously described (Cardozo-Pelaez et al., 2005, 1999). Briefly, RHSC were weighed and homogenized by sonication in 500 μL of perchloric acid (0.05 M) containing 3,4-dihydroxybenzylamine (DBA, 31 ng/mL). Sample filtrates were injected onto HPLC using an ESA Model 542 autosampler (ESA Chelmsford, MA). The mobile phase consisted of water:acetonitrile (9:1, vol/vol) containing 0.15 M monochloroacetic acid, 0.12 M sodium hydroxide, 0.60 mM EDTA, and 1.30 mM sodium octyl sulfate. An ultrasphere ODS column with a length of 25 cm and internal diameter of 4.6 mm was used for separation (Beckman Instruments, San Ramon, CA). The flow rate was kept at 1 mL/min (ESA Model 582 Solvent Delivery Module, Chelmsford, MA) and the column eluent was analyzed with an electrochemical detector (ESA Model 5600A CoulArray Detector, 3 ESA Model 6210 four channel electrochemical cells, Chelmsford, MA). Dopamine and DBA were monitored at 100 mV; HVA was monitored at 300 mV. The ratio of the peak heights produced by dopamine and its metabolite HVA to the peak height produced by DBA (internal standard) in the samples were used to obtain the hippocampus analyte levels from a calibration curve. Data was expressed as nanograms of analyte per gram of wet tissue weight (ng/g wt).

2.4. Focal embolic stroke

Animal procedures were approved by the University of Montana and Henry Ford Hospital Institutional Animal Care and Use Committees. Focal embolic stroke was established in adult male Wistar rats (Charles River Labs, Wilmington, MA) weighing 350–450 g, as described (Zhang et al., 1997a, 1997b). Assessments of infarct volumes, modified neurological severity score, foot-fault and adhesive tape removal were performed post stroke on days 1 and 7, as previously described (Zhang et al., 1997a, 1997b). Each test group contained a minimum of 8 randomized animals. Only animals that received a severe stroke (NSS >10) were included. For the dose-response study, four groups of 8 male Wistar rats/group were given 0 (vehicle control; saline), 0.1, 0.5, or 1 mg/kg/h methamphetamine via a 24 h continuous IV infusion starting immediately after embolic stroke on Day 0. For time course studies, groups of 8 randomized male Wistar rats received either 24-h I.V. infusion of saline or 1 mg/kg/h methamphetamine with administration beginning at either 6 or 12 h after stroke. Behavioral assessments including neurological severity scores, foot-fault and adhesive tape removal were performed on all rats by blinded observers at 24 h after stroke and again at 7 days after stroke, as described (Zhang et al., 1997a, 1997b). At 7 days after stroke, all rats were euthanized and infarct volumes were measured. Wortmannin (Sigma Chemical, St. Louis, MO) was mixed in sterile DMSO at 1.0 mg/mL and diluted in sterile saline. At 5 h post stroke, the animals were lightly anaesthetized using 1% isofluorane and 200 μL of a 15 μg/kg solution was injected into the tail vein (Noraberg et al., 1999; Zhang et al., 2007). One hour after the injection the animal received methamphetamine at 1.0 mg/kg/h or saline, as described above.

2.5. Phosphorylated AKT ELISA

Occluded rats were treated with IV infusion of 1.0 mg/kg/h methamphetamine or saline beginning 6 h after stroke. At 12 h post stroke all rats were euthanized, the brains removed, and each hemisphere dissected in ice-cold PBS. The cortex, hippocampus, and striatum from each hemisphere were homogenized and lysed in a buffer containing protease and phosphatase inhibitors (Sigma Chemical, St. Louis MO). Protein concentrations were determined by Bradford assay (Thermo Scientific, Rockford IL) and 100 μg of protein was loaded in triplicate into wells of a Surveyor IC phospho-AKT (S473) immunoassay plate (R&D systems, Minneapolis MN). Plates were processed according to manufacturer's protocol. Optical density was determined at 450 nm with a wavelength correction set at 540 nm.

2.6. TUNEL analysis

Twenty-four hours after embolic MCAO, rats from methamphetamine and saline treated groups were deeply anesthetized and perfused transcardially with 4% paraformaldehyde fixative. Brains were removed and post-fixed in 4% paraformaldehyde for 24 h at 4 °C. The tissue was then divided into 2 mm coronal sections using a rat brain matrix. Slices were processed, paraffin embedded, and sectioned at 7 μm. A 1 in 10 series from each rat was stained for Hematoxylin and Eosin (H&E). Using the H&E stained slides as a guide, 7 μm sections were mounted beginning approximately at bregma 1.6. Sections were matched for anatomical location using a rat brain atlas. A total of 6 slices per rat were mounted throughout the forebrain which were approximately 0.8 mm apart. Sections were stained using the DeadEnd Fluorometric TUNEL System (Promega, Madison WI) following the manufacturer's instructions. Slides were coverslipped using ProLong Gold antifade reagent with DAPI (Invitrogen, Carlsbad CA). TUNEL stained brain sections from 4 rats per treatment group were imaged with an Olympus SZX16 Research Stereomicroscope using Olympus DP Controller software (Olympus, Center Valley PA). Six sections per rat were matched for anatomical location as detailed above and then slides were blinded as to treatment group. Images were captured at 1×. Integrated Optical Density (IOD) of TUNEL staining was measured on the injured hemisphere of the brain section using ImagePro 6.2 software (ImagePro, Bethesda MD). The sum of the IOD from 6 sections was calculated per rat. Then treatment group averages were calculated from these sums.

2.7. Statistical analysis

All statistical analysis was performed using Prizm Software. To determine Gaussian (normal) distribution, a Kolmogorof–Smirnov test was performed on all data sets. Appropriate parametric analysis was performed on data sets containing two groups using an unpaired, two-tailed *T*-test (CI = 95%). Analysis on three or more data sets was done using one-way ANOVA with Tukey's post-hoc to determine statistical significance between groups. A $p < 0.05$ or less was considered significant.

3. Results

3.1. Methamphetamine reduces neuronal death after OGD

Neuronal injury, in the form of hypoxia/ischemia, was modeled *in vitro* by exposing rat organotypic hippocampal slice cultures (RHSC) to 60 min of oxygen–glucose deprivation (OGD). Immediately following OGD, cultures were transferred to media containing graded concentrations (100 μM–8 mM) of methamphetamine. Neuronal cell death was assessed by staining cultures with propidium iodide (PI), and measuring the relative fluorescence intensity at 24 h after OGD (Noraberg et al., 1999). Methamphetamine treatment resulted in a significant decrease in PI fluorescence, indicating increased neuronal survival over a broad dose range (100 μM–4 mM) with the greatest degree of protection observed within the CA1 region (Fig. 1a). In contrast, and consistent with the observation that high dose methamphetamine is neurotoxic, exposure to 8 mM methamphetamine not only failed to protect neurons, but significantly increased cell death throughout all regions.

We next investigated the time frame in which methamphetamine could be applied after injury and elicit a neuroprotective response. Using the lowest dose tested in the dose-response study (100 μM), methamphetamine was added to slice cultures at 2, 4, 8, and 16 h after OGD. Slices were again imaged at 24 h post-OGD. Significant increases in neuronal survival were observed in all three regions examined (CA1, CA3 and dentate gyrus), when methamphetamine was added to cultures up to 16 h after OGD (Fig. 1b). However, a moderate but non-significant decrease in protection was observed at the later time points, particularly within the CA1 region.

3.2. Methamphetamine is neuroprotective following transient focal embolic MCAO in rats

We further tested the hypothesis that methamphetamine is neuroprotective in an animal model of focal embolic stroke (Zhang et al., 1997a) Therapeutic efficacy of methamphetamine in this model was based on the blinded assessment of: 1) infarct volumes, 2) neurological severity scores (NSS), 3) foot-fault assessment, and 4) time to remove adhesive tape from both forepaws. There were no significant differences in behavioral outcomes between methamphetamine and saline treated rats on day 1 following stroke, indicating that all animals experienced a stroke of comparable severity (Fig. 2b–d). Only animals that experienced a severe stroke (NSS >10 on day 1) were included in the study.

A dose-dependent, neuroprotective effect was observed when animals were administered methamphetamine at 0.1, 0.5, or 1.0 mg/kg/h by continuous IV infusion for 24 h beginning immediately after stroke. Animals treated with 0.1 mg/kg/h did not show significant improvement compared to saline controls at 7 days post injury in any of the assessments (Fig. 2 a–d). However, animals treated with 0.5 or 1.0 mg/kg/h showed a significant reduction in infarct volumes, and significant improvement in behavioral outcomes based on NSS scores, and adhesive tape removal times compared to saline control animals. Although rats treated with 0.5 mg/kg/h failed to show improvement in the foot-fault assessment relative to saline controls, they did have significant improvements in NSS, adhesive tape removal and infarct volumes relative to saline controls. Animals treated with 1.0 mg/kg/h showed significant improvements in all four assessments compared to saline controls (Fig. 2c).

Based on these observations, we performed delayed treatment experiments using the 1.0 mg/kg/h dose to assess the practical clinical potential of methamphetamine as a neuroprotective agent. A significant reduction in infarct volume and a significant improvement in all behavioral assessments over saline controls were observed when the time of methamphetamine administration was delayed until 6 h after stroke (Fig. 2e–h). In contrast, reductions in infarct volumes were not significantly different from saline controls when treatment was delayed until 12 h after stroke (Fig. 2e). In addition, adhesive removal times did not differ between saline and methamphetamine treated animals when administration was delayed until 12 h after stroke (Fig. 2h). However, significant improvements in foot-fault and NSS assessments were observed in methamphetamine treated animals compared to saline controls at the 12-h time point (Fig. 2f and g).

3.3. Administration of low dose methamphetamine reduces apoptosis

Given the observed potential for methamphetamine to reduce cell death and improve functional outcomes, even when administration was initiated hours after injury, we tested the hypothesis that methamphetamine promotes cell survival by reducing apoptotic cell death. We first compared cleaved caspase 3 levels between saline treated RHSCs and cultures exposed to 100 μM methamphetamine following 60 min of OGD. Western blot analysis of cell lysates prepared 24 h after OGD revealed that methamphetamine treatment reduced cleaved caspase 3 levels by more than two-fold compared to saline treated cultures (Fig. 3a).

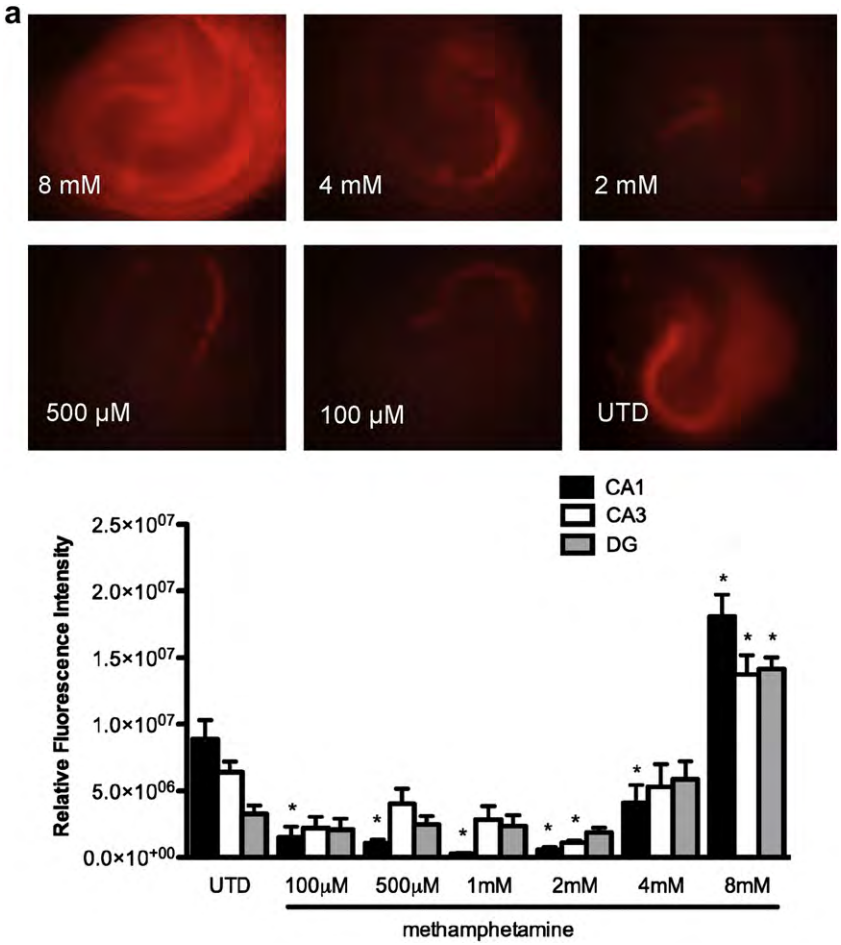

Fig. 1. Methamphetamine treatment promotes neuronal survival in RHSC exposed to OGD. (**a**) The dose-response of methamphetamine treatment in RHSC is presented. In the top panel, six representative images of RHSC stained with propidium iodide (PI) are shown. The images are labeled with the concentrations of methamphetamine that each respective slice culture was exposed to. The bottom panel shows a graphic representation of all slices tested. The concentrations of methamphetamine used in the respective dose groups are shown below the bars. All cultures were exposed to 60 min of OGD. Untreated (UTD) cultures were exposed to OGD but not to methamphetamine. The mean relative PI fluorescence intensities observed within the CA1 (black bars), CA3 (white bars) and dentate gyrus (DG; gray bars) are shown. (**b**) Again, images showing representative PI fluorescence after 60 min OGD are presented in the top panel. The times at which methamphetamine was added to the cultures are shown on the images. All cultures were exposed to 100 μM methamphetamine except for the untreated (UTD) group. The time at which methamphetamine was added to the cultures is shown below each group. The mean relative PI fluorescence intensities observed within the CA1 (black bars), CA3 (white bars) and dentate gyrus (DG; gray bars) are shown. Error bars represent mean ± SEM. Each bar represents a minimum of 7 slices; * = $p < 0.05$.

The ability of methamphetamine to block apoptosis was further examined in rats treated with saline or 1.0 mg/kg/h methamphetamine beginning at 6 h after embolic MCAO. Animals were euthanized 24 h after MCAO and brains were prepared for histological analysis. Apoptotic cells within the striatum and cortex were labeled by TUNEL staining and quantified by fluorescent microscopy. Methamphetamine treatment caused a significant reduction in TUNEL positive cells, with the greatest difference observed within the cortex (Fig. 3b). The observed differences in TUNEL staining correlated with TTC staining and infarct volumes (Fig. 2), suggesting methamphetamine mediates neuroprotection, in part, by blocking apoptosis.

3.4. The role of dopamine in methamphetamine-mediated neuroprotection

RHSCs provide a useful model due to the ease of experimental manipulation as well as the maintenance of cell populations and cytoarchitecture. Therefore, we used RHSCs to investigate the potential molecular mechanisms and signaling pathways associated with methamphetamine-mediated neuroprotection. D1 and D2 dopamine receptors are located within the hippocampus with D2 receptors found predominantly within the CA1 region and D1 receptors predominantly within the CA3 and dentate gyrus (Amenta et al., 2001a, 2001b; Yokoyama et al., 1995). However, to test the hypothesis that dopamine elicits a neuroprotective response in the RHSC model, we examined the potential of dopamine to reduce neuronal loss following OGD. Graded doses of dopamine (10 nM–1 mM) were added to slice cultures immediately following 60 min of OGD and PI fluorescence was measured 24 h later. A strong, dose-dependent improvement in neuronal survival was observed within the CA1, CA3 and dentate gyrus regions of cultures treated with 10 μM–1 mM dopamine (Fig. 4a). Significant improvement in neuronal survival was also observed in the CA3 and dentate gyrus regions of cultures treated with 10 nM dopamine. However, protection was not observed within the CA1 region following treatment with 10 nM dopamine.

The hippocampus receives dopaminergic input from the VTA. However, this connection is obviously severed in slice cultures. Therefore, we used HPLC analysis to validate the model and confirm that cultured hippocampal slices contained dopamine stores that could be released after seven days in culture. HPLC analysis

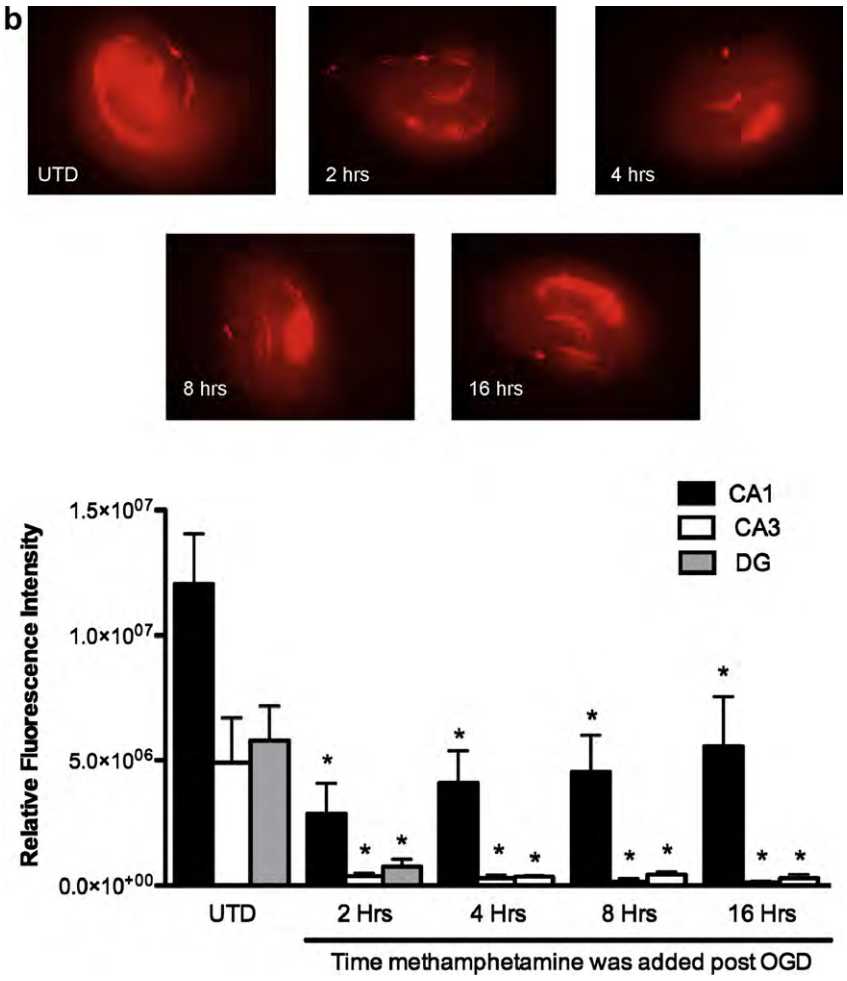

Fig. 1. (*continued*).

indicated that freshly prepared rat hippocampal slices contained approximately 6 ng of dopamine/g of total tissue weight. In addition, the presence of homovanillic acid (HVA), a product of dopamine metabolism, indicated active turnover of dopamine within freshly prepared slices (Fig. 4b). Although reduced in comparison with freshly prepared slices, dopamine and HVA were present in RHSCs after seven days in culture (Fig. 4c).

To determine which of the dopamine receptors (D1 like or D2 like) play a role in methamphetamine-mediated neuroprotection, we exposed RHSC to OGD and treated cultures with 100 μM methamphetamine in the presence of D1 (SCH23390; 10 μM) or D2 (Raclopride; 10 μM) dopamine receptor antagonists. The application of either antagonist in the absence of methamphetamine did not alter neuronal damage following OGD (data not shown). Each antagonist independently caused a significant decrease in methamphetamine-mediated neuroprotection, particularly within the CA1 and dentate gyrus regions (Fig. 4d). Interestingly, antagonism of D2 dopamine receptors alone caused a complete loss of protection within the CA3 region and antagonism of both receptor types was required to completely eliminate protection within the C1 region. However, protection was still observed within the dentate gyrus even when both antagonists were added.

3.5. Methamphetamine mediates neuroprotection via PI3K signaling

It has been proposed that dopamine receptor activation leads to neuroprotection through a PI3K signaling pathway that can involve both NMDA and AMPA receptors (Lee et al., 2002; Zou et al., 2005). We tested the hypothesis that methamphetamine-mediated neuroprotection was dependent, at least in part, on the activation of PI3K by adding the PI3K inhibitor LY294002 (5 μM), in combination with methamphetamine (100 μM) to RHSC after 60 min of OGD. LY294002 significantly reduced methamphetamine-mediated neuroprotection within the CA1 and dentate gyrus (Fig. 5). However, as with the independent antagonism of the D1 and D2 dopamine receptors, inhibition of PI3K did not completely block the neuroprotective effect of methamphetamine, suggesting that additional signaling mechanisms may be involved.

These *in vitro* findings were confirmed *in vivo* by treating rats with methamphetamine in combination with the PI3K inhibitor wortmannin (15 μg/kg). Wortmannin treatment completely blocked reduction in infarct volumes and improvements in all of the behavioral assessments previously observed with methamphetamine administration (Fig. 2e–h). Treatment of rats with saline in combination with wortmannin did not increase infarct volumes or worsen behavioral assessments. These data strongly suggest that methamphetamine-mediated neuroprotection is dependent, in part, on PI3K signaling.

3.6. Methamphetamine increases activation of AKT

Activation of PI3K results in the phosphorylation and activation of AKT. In turn, activated AKT (pAKT) phosphorylates multiple targets to initiate a cascade of events that prevent apoptosis and promote cell survival (Plas et al., 2001; Uchiyama et al., 2004).

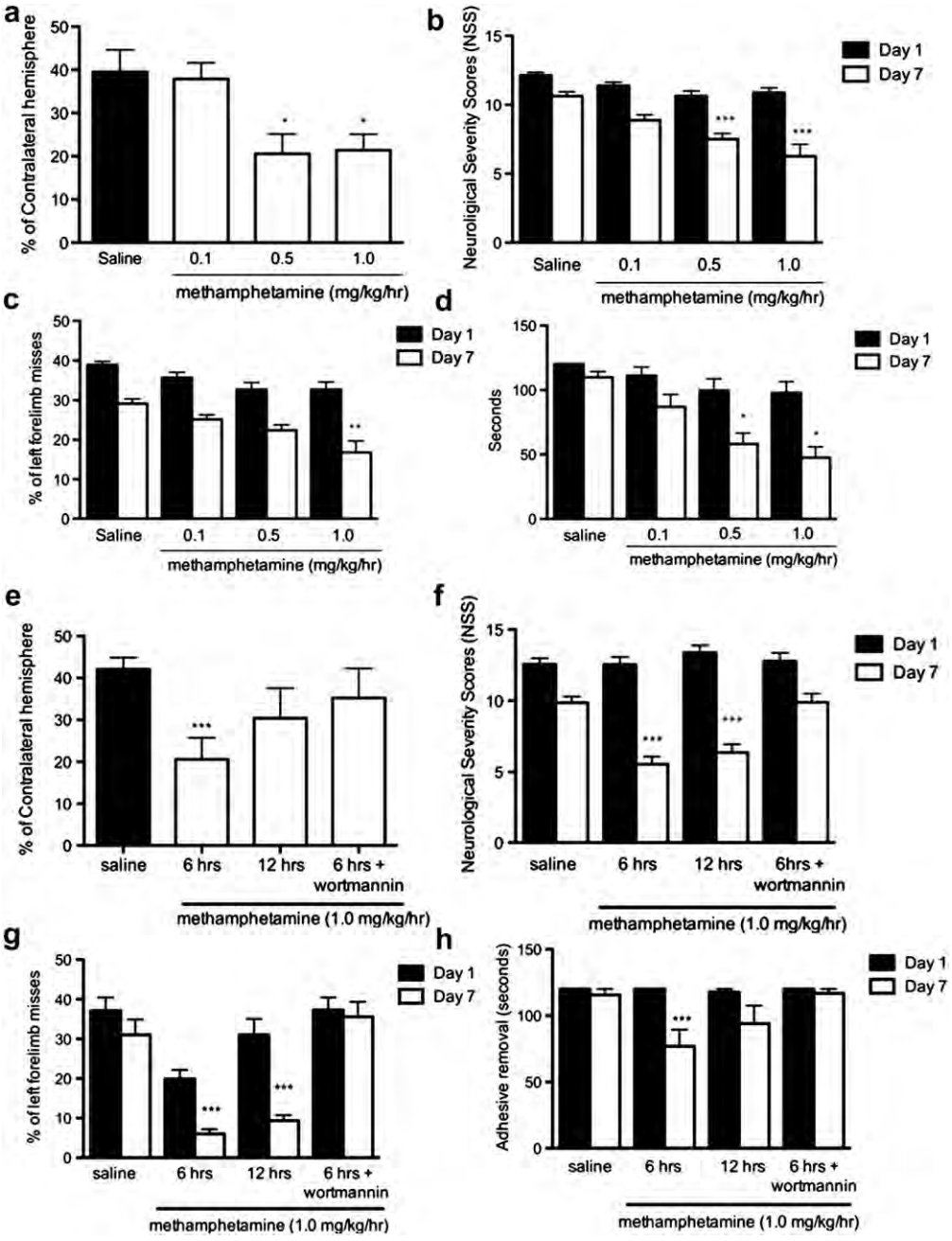

Fig. 2. Methamphetamine treatment reduces infarct volumes and improves functional outcomes following rat embolic MCAO. A 4 cm clot was delivered via a catheter to the opening of the middle cerebral artery of adult, male, Wistar rats. Animals were treated with saline or graded doses of methamphetamine (0.1, 0.5, or 1.0 mg/kg/h) by femoral vein infusion for 24 h immediately after clot placement (**a**–**d**). For the time course study, rats were treated with 1.0 mg/kg/h for 24 h beginning either 6 or 12 h after MCAO (**e**–**h**). Rats were euthanized at 7 days after MCAO and brain sections were stained with TTC to measure infarct volumes (**a** & **e**). Neurological severity scores (NSS) (**b** & **f**), foot-fault (**c** & **g**) and adhesive removal times (**d** & **h**) were assessed at 1 (black bars) and 7 (white bars) days after MCAO. Error bars represent mean ± SEM. Each bar represents a minimum of 8 rats; (* = $p < 0.05$, ** = $p < 0.01$; *** = $p < 0.001$; comparisons were made relative to saline treated animals at 7 days post injury).

Therefore, we examined the possibility that methamphetamine treatment elicits the activation of AKT. Western blot analysis of AKT and pAKT within RHSCs confirmed that treatment with 100 μM methamphetamine significantly increased the ratio of pAKT to AKT (Fig. 6a & b). The increase in pAKT was blocked by the addition of the PI3K inhibitor LY29002 (10 μM), or the D1 antagonist SCH23390 (10 μM) and the D2 antagonist raclopride (10 μM), alone or in combination. These data suggest that methamphetamine promotes cell survival through a PI3K/AKT signaling pathway that is dependent on dopamine activation of D1 and D2 receptors.

The RHSC data was again confirmed *in vivo* by measuring region specific levels of pAKT within the injured hemisphere of rats that had received an embolic stroke. Methamphetamine administration (1 mg/kg/h) was started 6 h after stroke and tissue samples were collected 6 h later (12 h after stroke). The cortex, hippocampus, and striatum were dissected out and pAKT ELISA analysis was performed on methamphetamine and saline treated controls. A significant increase in pAKT was observed in all three regions sampled from methamphetamine treated animals relative to saline controls (Fig. 6b).

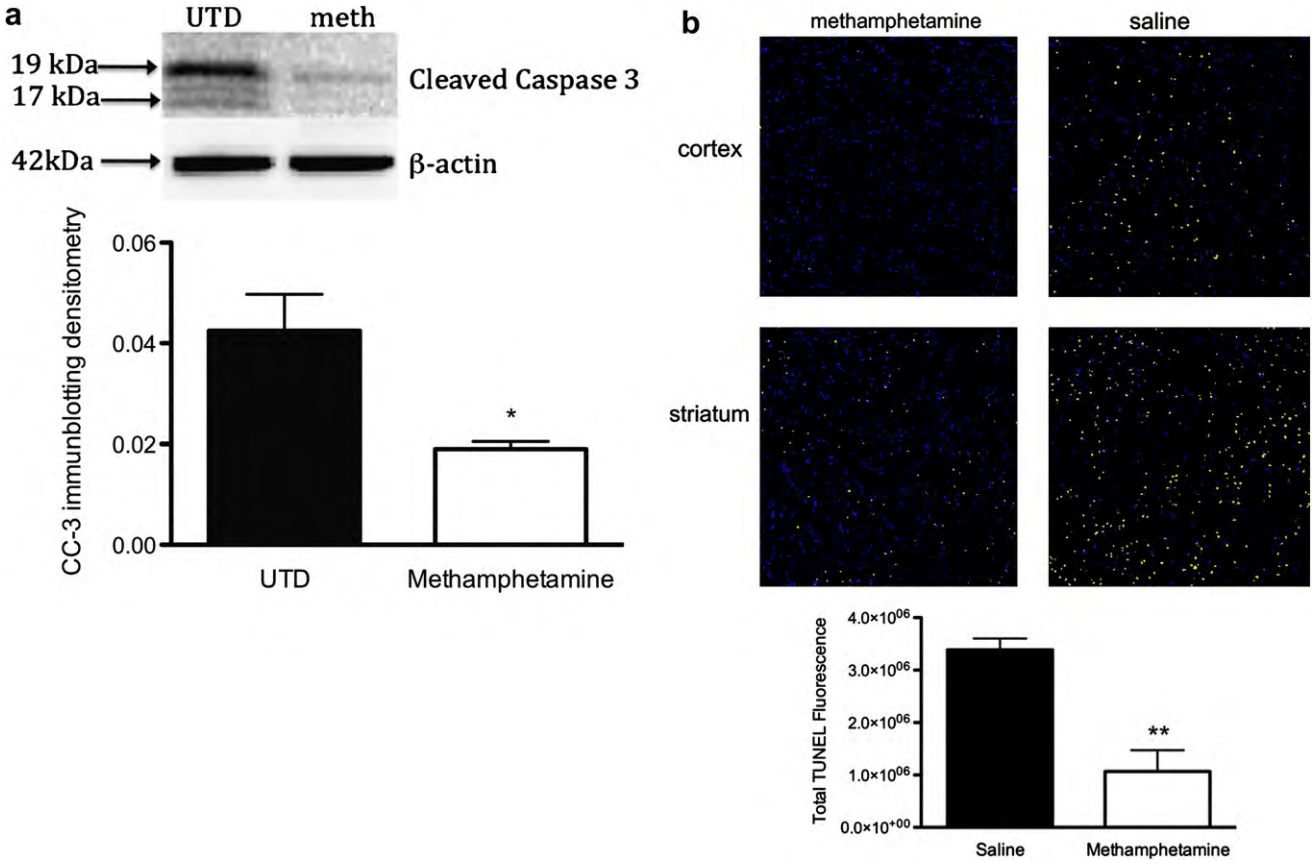

Fig. 3. Treatment with low dose methamphetamine reduces apoptotic cell death. (**a**) The level of apoptotic cell death within RHSC exposed to 60 min of OGD was determined by Western blot analysis of cleaved caspase 3. Three separate lysates were prepared by combining five separate slices/sample 24 h after OGD. Cultures were either untreated (UTD) or exposed to 100 μM methamphetamine. Each lysate was run in duplicate. The mean densitometry values normalized to b-actin are presented. * = $p < 0.5$ relative to untreated (UTD) saline controls (**b**) Paraffin embedded sections from rats that received embolic stroke were prepared and stained with TUNEL to label apoptotic cells within the cortex and striatum. The top panel shows representative images from methamphetamine and control treated rats. Sections were dual stained with TUNEL (green) to label apoptotic cells and DAPI (blue) to label all nuclei. Total TUNEL fluorescence within the striatum and cortex combined is show in the graph beneath the images. * = $p < 0.05$; ** = $p < 0.01$.

4. Discussion

Although methamphetamine is FDA approved and has been safely used for the treatment of attention deficit disorder in children for many years, an overwhelming number of studies have focused on the neurotoxic potential of this drug (Krasnova and Cadet, 2009). These neurotoxicity studies have typically employed repeated, high doses of 10 mg/kg or greater, which replicate conditions of abuse. Under these conditions, methamphetamine clearly exerts a neurotoxic effect. Indeed, our data here indicate that the addition of methamphetamine at a concentration of 8 mM increased neuronal death in RHSCs following OGD. However, we have also demonstrated that low to moderate doses of methamphetamine can clearly induce a robust neuroprotective response. Consistent with our findings, Smith et al. (2007) reported that treatment of RHSCs with 1–100 μM methamphetamine reduced neuronal loss following exposure to 5 μM NMDA. In addition, a recent 5 year retrospective case study analysis of 583 patients with severe TBI indicated that patients who tested positive for methamphetamine at the time of admission to the emergency room had a 25% improvement in survival (O'Phelan et al., 2008). Collectively, these data provide converging support for the hypothesis that low doses of methamphetamine can be neuroprotective when administered as an acute treatment following injury.

Most neuroprotective agents evaluated to date have required administration within a very short period of time following injury. This short therapeutic window has severely limited their application potential. We demonstrate here that significant improvement in functional outcomes can be obtained even when low dose methamphetamine is administered as late as 12 h after embolic MCAO. These data suggest that many of the deleterious pathways initiated under conditions of stroke may be blocked or reversed within this therapeutic window.

Our data indicate that dopamine plays a major role in methamphetamine-mediated neuroprotection. The fact that up to 1 mM dopamine was neuroprotective in the RHSC/OGD model was somewhat surprising, given that high doses of dopamine have been reported to induce neurotoxicity. It is possible that within the conditions of the slice culture system, dopamine diffusion through the media does not achieve toxic levels. However, it is noteworthy that the addition of 8 mM methamphetamine was neurotoxic in this culture model (Fig. 1a). These observations in combination would suggest that in this slice culture model, dopamine may not make a major contribution to methamphetamine-mediated neurotoxicity. Methamphetamine also induces the release of norepinephrine and serotonin. One or both of these neurotransmitters could be responsible for the neurotoxicity associated with 8 mM methamphetamine.

Injury from hypoxia/ischemia involves a cascade of pathogenic processes. Direct and indirect interactions of dopamine receptors with NMDA and AMPA receptors may modulate excitatory currents (Lee et al., 2002; Liu et al., 2006). In addition, dopamine strongly

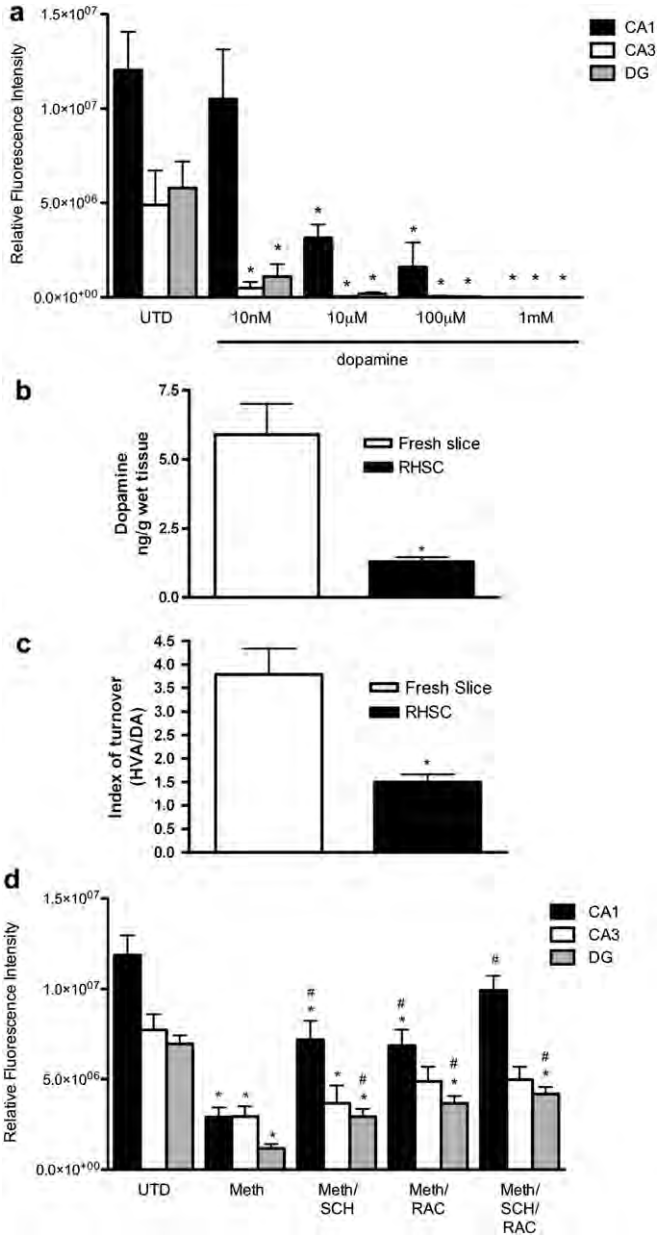

Fig. 4. Dopamine plays a major role in methamphetamine-mediated neuroprotection in RHSC exposed to OGD. (**a**) RHSC exposed to 60 min of OGD were untreated (UTD) or incubated with graded concentrations of dopamine (10 nM–1 mM) for 24 h after injury. Slices were stained with PI to label damaged neurons. The relative intensity of PI fluorescence is presented as a measure of neuronal damage (* = $p < 0.05$ relative to UTD). HPLC analysis of lysates from RHSC was performed to measure dopamine (**b**) and the ratio of homovanillic acid (HVA)/dopamine (DA) (**c**) in freshly prepared slices (white bars) and from slices after 7 days in culture (black bars) * = $p < 0.05$; $n = 6$. (**d**) RHSC exposed to 60 min OGD were untreated (UTD) or cultured with methamphetamine (meth, 100 μM) alone or with methamphetamine in combination with the D1 dopamine receptor antagonist SCH23390 (SCH, 10 μM) or the D2 dopamine receptor antagonist raclopride (RAC, 10 μM). * denotes significance between UTD vs. all groups. # denotes significance between methamphetamine alone vs. all groups. * = $p < 0.05$; # = $p < 0.05$; each bar represents a minimum of 6 slices.

suppresses glutamatergic hippocampal and entorhinal neurotransmission by activation of presynaptic D1 dopamine receptors (Behr et al., 2000). Dopamine may also modulate inhibitory neurotransmission in a post-synaptic manner through AKT signaling. For example, AKT mediated phosphorylation of the β2 subunit of the GABA-A receptor results in increased density of GABA-A receptors within the post-synaptic membrane, leading to enhanced inhibitory signaling (Wang et al., 2005, 2003).

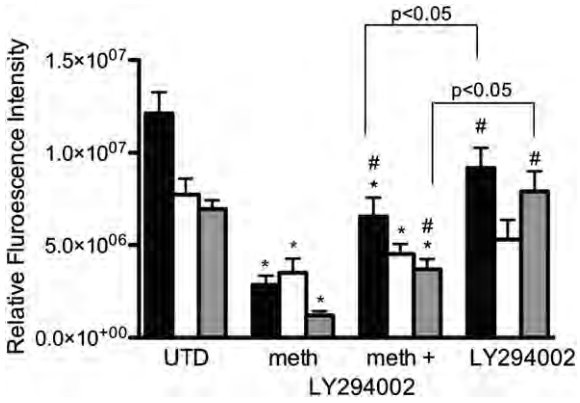

Fig. 5. Inhibition of PI3K reduces methamphetamine-mediated neuroprotection. RHSC exposed to 60 min of OGD were untreated (UTD) or cultured in the presence of 1) methamphetamine (100 μM); 2) the PI3K inhibitor LY294002 (5 μM); or 3) methamphetamine combined with LY294002. Damaged neurons were stained with PI and values are presented as relative fluorescence intensity. * denotes significance relative to UTD samples ($p < 0.05$). # denotes significance between methamphetamine alone vs. all groups. Each bar represents a minimum of 10 slices. Black bars = CA1 region, White bars = CA3 region and gray bars = dentate gyrus.

Activation of the D1 and D2 dopamine receptors has also been linked to PI3K signaling (Lee et al., 2002; Zou et al., 2005). Our data indicate that methamphetamine initiates a cell survival-signaling cascade that involves both D1 and D2 dopamine receptors, PI3K, and AKT. AKT is a critical, pro-survival kinase that suppresses a number of apoptotic mechanisms leading to neuronal survival (Datta et al., 1997; Plas et al., 2001). Previous studies involving hypoxia-ischemia have shown that pAKT suppresses activation of mitochondrial mediated cleaved caspase 9 in neurons (Dhanasekaran et al., 2008; Uchiyama et al., 2004). Further studies have determined pAKT inactivates pro-apoptotic BAD by phosphorylating BAD binding protein, 14-3-3. The binding of 14-3-3 to BAD blocks the formation of the BAD–Bcl-xl complex and allows Bcl-xl to promote cell survival (Jeong et al., 2008; Koh et al., 2008; Uchiyama et al., 2004). pAKT also activates inhibitors of apoptosis, particularly XIAP (Katz et al., 2001; Kwon et al., 2006; Wang et al., 2004). While effectively blocking apoptosis in neurons, pAKT also serves to promote cell survival in part by modulating the forkhead transcription factor and tumor suppressor p53 (Brunet et al., 2001; Fiskum et al., 2004; Kiang and Tsen, 2006; Wang et al., 2004). pAKT directly phosphorylates forkhead at Thr24, Ser256 and Ser319, resulting in nuclear export and inhibition of transcription factors, ultimately establishing conditions that lead to cell survival (Arden, 2004; Brunet et al., 1999). To modulate p53 activity, pAKT phosphorylates MDM2, which binds to p53, inhibiting its accumulation by targeting it for ubiquitination and degradation (Chehab et al., 1999; Honda et al., 1997). Together, these data indicate that low to moderate doses of methamphetamine can provide neuroprotection, at least in part, through a D1/D2-PI3K-AKT signaling pathway.

In addition to dopamine, methamphetamine also increases synaptic serotonin levels, which may also contribute to neuroprotection. Neuroimaging studies of chronic methamphetamine abusers show a decrease in serotonin transporters in the midbrain, caudate, putamen, hypothalamus, thalamus, the orbitofrontal, temporal, and cingulate cortices (Thrash et al., 2009). However, moderate post-synaptic activation of serotonin receptor 5-HT_{1a} prevents ischemic brain damage and acute subdural hematoma (Klisch et al., 2003; Ramos et al., 2004). In addition, the administration of Fluoxetine, a selective serotonin reuptake inhibitor, significantly reduced infarct volumes and neurological impairment in rats when delivered up to 9 h after MCAO (Lim et al., 2009).

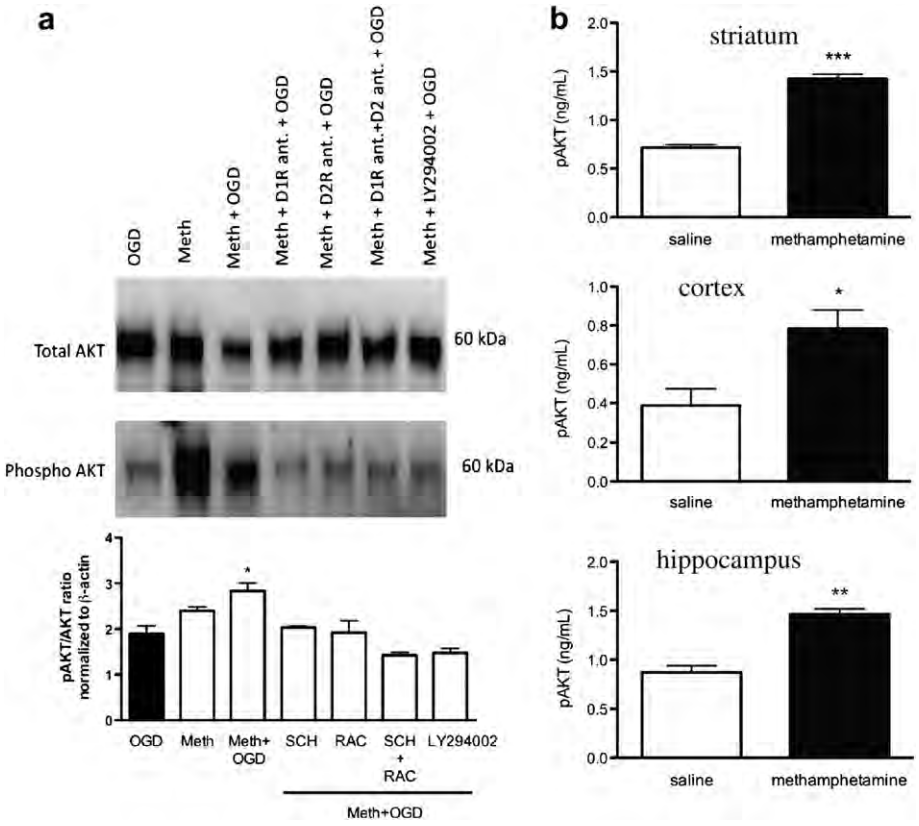

Fig. 6. Methamphetamine increases phosphorylation of AKT (**a**) A representative Western blot image showing changes in the levels of phosphorylated AKT (pAKT) in lysates from RHSC exposed to 60 min of OGD. Slice cultures were treated immediately after OGD with 100 μM methamphetamine alone or in combination with the PI3K inhibitor LY294002 (10 μM), the D1R antagonist SCH23390 (SCH, 10 μM), the D2R antagonist raclopride (RAC, 10 μM) or both the D1R and D2R antagonists. The bar graph shows mean values ± SEM normalized to b-actin. Error bars represent mean. Each bar represents a minimum of 7 slices $^{*} = p < 0.05$. (**b**) The levels of pAKT within the brains of saline and methamphetamine treated rats were measured 12 h after MCAO and 6 h after methamphetamine administration (1 mg/kg/h) was begun. pAKT levels were assessed by ELISA analysis. Lysates were prepared from striatum, cortex and hippocampus of 3 rats/treatment group. $^{*} = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.001$.

Similar to the data we present here with methamphetamine, Lim et al. (2009) observed that significant reduction in infarct volumes were lost when Fluoxetine administration was delayed until 12 h after MCAO (Lim et al., 2009). However, in contrast to this study, we observed significant improvements in NSS assessments when methamphetamine administration was delayed until 12 h after MCAO. Interestingly, Lim et al. (2009) indicated that blocking reuptake of serotonin significantly reduced neutrophil infiltration, microglial activation, and reduced pro-inflammatory NF-kappaB activity (Lim et al., 2009).

Norepinephrine is also released by methamphetamine and may activate pro-survival pathways, as well, through nitric oxide signaling that increases phosphorylation of PI3K, and increases expression of pro-survival BDNF, pCREB, pAKT and pMAPK (Patel et al., 2010). In addition, Junker et al. (2002) presented data suggesting that norepinephrine and the b_2-adrenoreceptor agonist clenbuterol produced a moderate reduction in infarct volumes (Junker et al., 2002). Thus, in light of these observations, it would be reasonable to hypothesize that methamphetamine may induce neuroprotection through multiple catecholaminergic pathways.

Finally, it is worth noting that a number of labs have reported, in contrast to our data, that treatment with amphetamines can induce a decrease in AKT phosphorylation (Beaulieu et al., 2009, 2004; Chen et al., 2007). This discrepancy with our observations may be explained by the differences in culture systems. Many of these other studies involved the use of non-neuronal, transformed cell lines, synapotosomes or the activation of D2 dopamine receptors alone. Furthermore, these studies examined phosphorylation of AKT at Thr 308. In contrast, we monitored AKT phosphorylation at Ser 473 as phosphorylation at this site has been associated with neuroprotection (Shehadah et al., 2010; Zhang et al., 2010). However, one of the most compelling explanations for the differences observed in our studies is that we examined the effects of methamphetamine under conditions of hypoxia/ischemia. It is intriguing to consider the possibility that the neuroprotective effects of methamphetamine we observed required the coupling or cooperative activation of multiple dopamine and glutamate receptors. Under stroke conditions, this coupled activation of receptor combinations could induce modified signaling pathways relative to non-stroke conditions. This possibility is worthy of further investigation and suggests that a different paradigm, which takes into account the physiological conditions associated with a specific injury or disease state, may be required for the development of effective neuroprotective agents.

Acknowledgments

This work was supported through grants: MBRCT09-51 and R01AG031184-01.

We wish to thank Annela Rova for her excellent technical assistance.

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Treatment with low-dose methamphetamine improves behavioral and cognitive function after severe traumatic brain injury

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BACKGROUND: Methamphetamine increases the release and blocks the reuptake of dopamine. The moderate activation of dopamine receptors may elicit neuroprotective effects. We have recently demonstrated that low doses of methamphetamine reduce neuronal loss after ischemic injury. On the basis of this finding, we hypothesized that methamphetamine could also prevent neuronal loss and improve functional behavior after severe traumatic brain injury (TBI).

METHODS: The rat lateral fluid percussion injury model was used to generate severe TBI. Three hours after injury, animals were treated with saline or methamphetamine. Neurological severity scores and foot fault assessments were used to determine whether treatment enhanced recovery after injury. The potential for methamphetamine treatment to improve cognitive function was assessed using the Morris water maze. Forty-eight hours after injury, paraffin-embedded brain sections were TUNEL stained to measure apoptotic cell death. Sections were also stained with antibody to doublecortin to quantify immature neurons within the dentate gyrus.

RESULTS: Treatment with low-dose methamphetamine significantly reduced both behavioral and cognitive dysfunction after severe TBI. Methamphetamine-treated animals scored significantly lower on neurological severity scores and had significantly less foot faults after TBI compared with saline-treated control rats. Furthermore, methamphetamine treatment restored learning and memory function to near normal ability after TBI. At 48 hours after injury, apoptotic cell death within the hippocampus was significantly reduced, and the presence of immature neurons was significantly increased in methamphetamine-treated rats compared with saline-treated controls.

CONCLUSION: Treatment with low-dose methamphetamine after severe TBI elicits a robust neuroprotective response resulting in significant improvements in behavioral and cognitive functions. (*J Trauma Acute Care Surg*. 2012;73: S165–S172. Copyright © 2012 by Lippincott Williams & Wilkins)

KEY WORDS: Traumatic brain injury; neuroprotection; methamphetamine.

Traumatic brain injury (TBI) affects more than 1.5 million people in the United States each year.[1,2] This results in more than 5 million individuals living with permanent motor dysfunction and cognitive impairment.[3] Currently, there is no treatment available to prevent neuronal loss after TBI. Although patients are given routine medical intervention to stabilize physiological function, the neurological damage remains untreatable. As TBI numbers increase from population growth and ongoing military operations, it becomes imperative to develop novel treatments that prevent neuronal damage and loss after TBI. However, the development of effective neuroprotective agents has been challenging, with more than 150 compounds failing in clinical trials. The reasons for these drug failures involve multiple factors. Although TBI activates a cascade of neuropathologies, many of the drugs tested to date have focused on single targets and have thus proven insufficient. In addition, many of the drugs tested have had a limited therapeutic window or have moved to clinical trials with insufficient preclinical data to support the dosing regimens used in humans.

We have recently identified a potential neuroprotective agent that addresses many of the limitations observed with previous compounds. We have reported that low-dose methamphetamine is neuroprotective within the context of an embolic stroke model.[4] This finding was unexpected given the large body of evidence describing the neurotoxic potential of methamphetamine. However, methamphetamine-mediated neurotoxicity is associated with high, repetitive doses. This results in multiple neuropathological events that ultimately lead to the loss of dopaminergic nerve terminals throughout the ventral tegmental area, substantia nigra, hippocampus, prefrontal cortex, and cortex.[5–7] In contrast, the administration of low-dose methamphetamine proved to be neuroprotective, even when treatment was delayed until 12 hours after injury.[4] We further demonstrated that methamphetamine mediates neuroprotection through the dopamine-dependent activation of a PI3 kinase–AKT signaling pathway that promotes neuronal survival through multiple targets.[4]

On the basis of the neuroprotective effects that we observed with the ischemic model, we hypothesized that methamphetamine could produce a similar neuroprotective effect after TBI. To test this hypothesis, we used the lateral fluid percussion injury model to produce a severe TBI in rats. Animals were treated with low-dose methamphetamine beginning 3 hours after injury. Behavioral assessments were measured every 7 days

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This study was presented at the Advanced Technology Applications for Combat Casualty Care Conference in Fort Lauderdale, Florida, August 15–18, 2011.

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DOI: 10.1097/TA.0b013e318260896a

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for a month, and cognitive function was assessed with the Morris water maze beginning on day 24 after injury. Histological analysis was performed on brain sections processed 48 hours after injury. Treatment with methamphetamine significantly improved both behavioral and cognitive function. Histological analysis revealed that treatment with low-dose methamphetamine significantly reduced apoptotic cell death within the hippocampus and increased the number and apparent health of immature neurons within the dentate gyrus after severe TBI.

METHODS

TBI Procedure

All procedures were approved by the University of Montana Institutional Animal Care and Use Committee. The lateral fluid percussion injury model was used to produce severe TBI as previously described.[8] Adult, male, Wistar rats (mean weight,400 g) were obtained from Charles River Laboratories (Wilmington, Mass) and housed in a temperature-controlled environment with a 12-hour light–12-h dark cycle and constant access to food and water. Rats were distributed randomly into four experimental treatment groups as follows: (1) saline-treated injured rats (n = 11), (2) methamphetamine-treated injured rats (n = 8), saline-treated uninjured sham controls (n = 8), and methamphetamine-treated uninjured sham controls (n = 7). Animals received a 5-mm trephination over the right hemisphere. Injury was induced by depressing the dura with 1.8 atm to 2.2 atm of pressure during a 20-ms time frame. Pressure was generated with an FPD 302 fluid percussion TBI device (Amscien Instruments, Richmond, Va). Immediately after injury, animals typically experienced a brief period of apnea (1–5 minutes), during which they were manually ventilated and provided supplemental oxygen (13% of the animals failed to survive the immediate, postinjury apnea). Animals exhibited an average righting time of 25 to 35 minutes (as determined by the animal being able to roll from its side and bear weight on all four limbs). The severity of injury was based on neurological severity scores (NSSs) assessed at 24 hours after injury (1–4 light injury, 5–9 moderate injury, and 10–15 severe injury). Inclusion into the study was limited to animals with an NSS higher than 10 (13% of injured animals received an NSS of less than 10). Sham-treated animals underwent the same surgical procedure but did not receive a TBI. At 3 hours after injury, animals received an intravenous bolus injection of saline or methamphetamine (0.845 mg/kg). This injection was timed to coincide with the initiation of a continuous intravenous infusion for a 24-hour period. Continuous infusion was accomplished by placing an Alzet pump (2001D, 6.7 μL/hour; Alzet Corp, DURECT Corp., Cupertino, CA) containing methamphetamine or saline into the right inguinal crease. The pumps were connected to a short piece of flexible P50 tubing that was filled with sterile saline. The tubing was inserted into the femoral vein as previously described.[4] The pumps were implanted 1 hour before delivery of the bolus dose, as this was the time required for the drug to travel from the pump and reach the femoral vein. Methamphetamine-treated animals received continuous intravenous infusion of the drug at 0.5 mg kg^{-1} h^{-1} for 24 hours. Control animals underwent the same procedure but received saline. NSS and foot fault assessments were conducted on days 1, 7, 14, 21, and 30 after injury as previously described.[4] Behavioral assessments were conducted by a trained, blinded observer.

For the assessment of cognitive function, the Morris water maze (MWM) was used to assess cognitive function (learning and memory) after severe TBI. Testing was performed as previously published by Choi et al.[9] The learning phase began on day 24 after injury and lasted for 5 days. Animals experienced four 2-minute training sessions per day. Latency, or the time required to locate the escape platform, was recorded and averaged for the four trials each day. On day 6, memory was assessed by removing the escape platform and by quantifying the time each animals spent searching for the platform in the target quadrant. The probe trial lasted a total of 60 seconds. Anymaze software (Stoelting [Stoelting Co., Wood Dale, IL] was used to capture and analyze the track paths and latency times).[9] Animals were distributed randomly amoung treatment groups, and MWM data were collected by a trained, blinded observer.

Histological Analysis

Forty-eight hours after TBI, a subset of injured animals was perfused with 4% paraformaldehyde by transcardiac puncture. Brains were postfixed in 4% paraformaldehyde for 24 hours at 4°C and cut into 2-mm coronal sections using a rat brain matrix. Slices were processed, paraffin embedded, and sectioned at 7 μm. Sections were mounted on slides and dried overnight at 37°C then deparaffinized in xylene and dehydrated through a graded series of ethanol. TUNEL-positive cells were labeled using the DeadEnd Fluorometric TUNEL System (Promega, Madison, Wis). Nuclei were counterstained with DAPI (Invitrogen, Carlsbad, Calif). Sections were imaged on an iCys Research Imaging Cytometer (CompuCyte, Westwood, Mass). Four saline-treated and three methamphetamine-treated animals were examined for TUNEL. Four brain sections were examined per animal at positions −3.3, −4.3, −5.3, and −6.3 relative to the bregma. The sum of all TUNEL-positive cells, in the four sections, in each rat was recorded. The average number of TUNEL positive cells per group is reported. Doublecortin (DCX)-positive neurons were labeled with rabbit anti-DCX antibody (AbCam, Cambridge, Mass). After deparaffinization and rehydration, antigen retrieval in citrate buffer was performed. Slides were then blocked in 4% normal goat serum and incubated overnight at 4°C with anti-DCX antibody diluted 1:5000. Sections were then reacted with Alexa 568–conjugated goat antirabbit IgG secondary antibody (Molecular Probes, Eugene, Ore) diluted 1:300. Three separate slices (140 μm apart) from three saline and three methamphetamine-treated rats were stained and analyzed for DCX labeling. Slices were imaged on an Olympus FV1000 confocal microscope. Integrated optical density of TUNEL and DCX staining was quantified using ImagePro 6.2 software (ImagePro, Bethesda, Md).

Statistical Analysis

All data were analyzed using Prizm software (GraphPad, La Jolla, CA). To determine Gaussian (normal) distribution, a Kolmogorof-Smirnov test was performed on all data sets. Appropriate parametric analysis was performed on data sets containing two groups using an unpaired, one-tailed *t* test (95% CI). Analysis on data sets with more than two groups was performed using oneway analysis of variance with Tukey's post hoc to determine

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statistical significance between groups. A $p < 0.05$ or less was considered significant.

RESULTS

Methamphetamine Treatment Prevents Neuronal Apoptosis After TBI

The lateral fluid percussion injury model has been used for more than two decades to study TBI. We used this well-established model to test the hypothesis that low-dose methamphetamine is neuroprotective in the context of severe TBI. Neuronal cell death occurs in two waves after TBI. The initial injury results in rapid, necrotic cell death because of physical damage. Events associated with this first wave lead to the release of factors that contribute to the secondary wave of death, characterized by apoptotic cell death.[10] This second wave progresses during a much longer time window and thus represents a critical target of intervention for treating TBI. Therefore, we examined the potential of methamphetamine to limit or to reduce apoptotic death after severe TBI. After severe injury, rats received an intravenous injection of methamphetamine at a dose of 0.845 mg/kg, followed by continuous intravenous infusion of

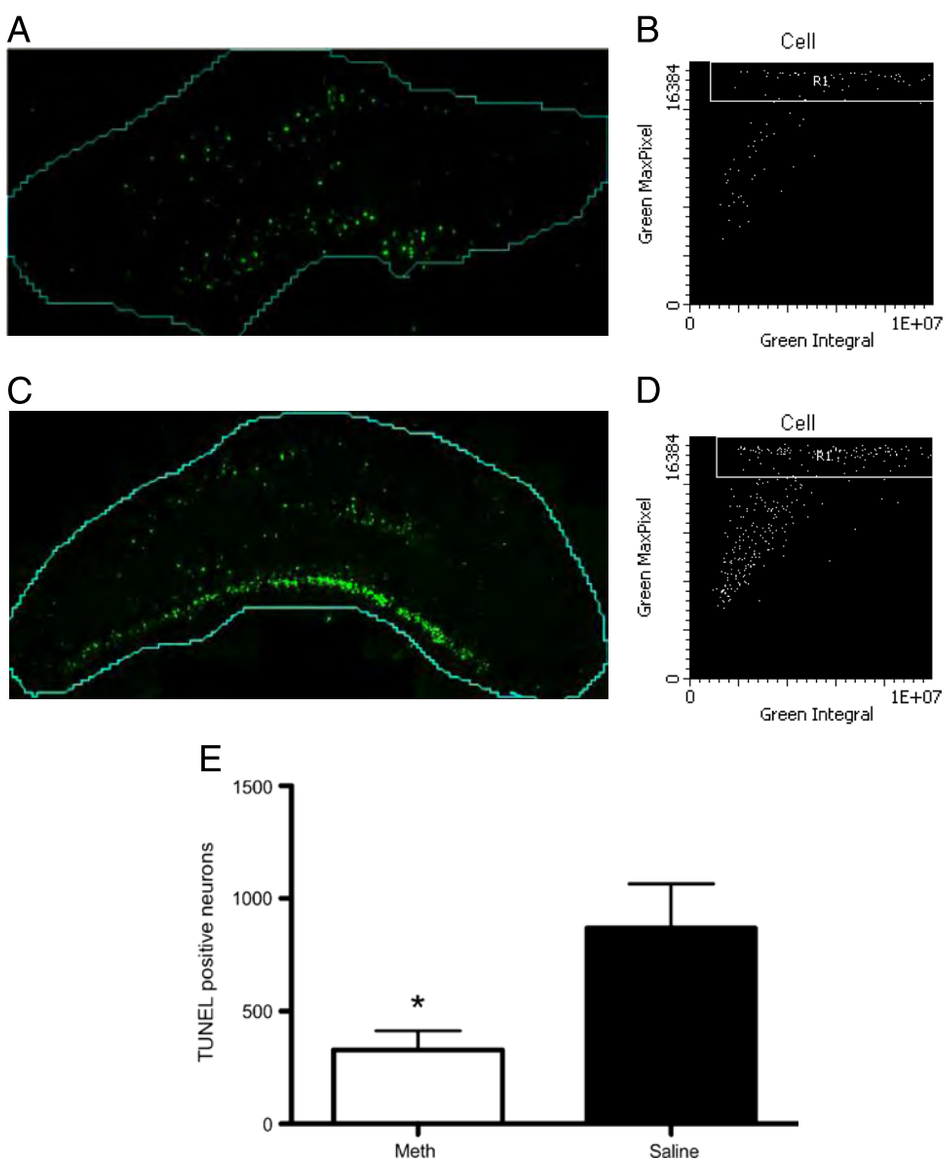

Figure 1. Low-dose methamphetamine reduces apoptosis in the hippocampus of animals with TBI. Methamphetamine-treated animals showed a significant decrease in TUNEL-positive, apoptotic neurons 48 hours after injury (mean, 869 TUNEL-positive neurons in saline-treated animals vs. 329 in methamphetamine-treated animals). TUNEL-positive cells were counted on a laser scanning cytometer. (*A*) Representative image from a methamphetamine-treated animal. (*B*) Representative scatter plot of the counted TUNEL-positive neurons. (*C* and *D*) TUNEL-positive cells within the injured hippocampus and a representative scatter plot from a saline-treated animal. (*E*) Graphical representation of TUNEL-positive neurons in both groups. *$p < 0.05$, two-tailed *t* test, n = 4.

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0.5 mg kg^{-1} h^{-1} for 24 hours. Control animals received injections of saline. Animals were killed 48 hours after the injury, and paraffin-embedded brain sections were stained with the TUNEL method to label apoptotic cells. TUNEL-positive cells within paraffin-embedded sections from injured rats were quantified using a laser scanning cytometer. Treatment with methamphetamine after severe TBI significantly reduced apoptosis within the hippocampus of the injured hemisphere (Fig. 1).

Low-Dose Methamphetamine Improves Functional Behavior After Severe TBI

NSS assessments were used to measure behavioral function and to quantify the potential therapeutic effect of methamphetamine after severe TBI. At 24 hours after injury, there were no significant differences in NSS between saline- and methamphetamine-treated animals (Fig. 2). These data indicate that all animals received an injury of equivalent severity. However, by 7 days after injury, methamphetamine-treated animals showed a significant improvement in behavioral function. This trend continued throughout the study, with methamphetamine-treated animals ultimately demonstrating behavioral function that was similar to uninjured sham controls. In contrast, saline-treated animals demonstrated a slight improvement on days 7 and 14 but failed to show additional improvement and remained significantly different from methamphetamine-treated and uninjured controls on day 30 (Fig. 2).

The same cohort of animals underwent foot fault assessment to quantify sensory and fine motor control as a second measure of behavioral function. Again, as with the NSS assessments, no significant differences were observed on day 1 after injury, further confirming that all rats received equivalent injuries. Unlike the NSS measure, no significant differences were observed between methamphetamine- and saline-treated animals on day 7 (Fig. 3). However, by day 14, methamphetamine-treated animals

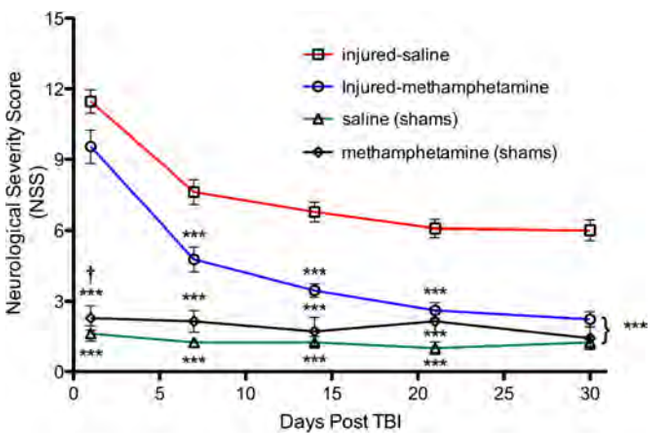

Figure 2. Low-dose methamphetamine treatment significantly improved functional behavior. NSSs are presented for injured methamphetamine-treated animals (*blue line*), injured saline-treated animals (*red line*), methamphetamine-treated sham controls (*black line*), and saline-treated sham controls (*green line*). NSS was assessed weekly for up to 30 days; n = 13 for saline- and methamphetamine-treated rats, n = 8 for sham controls. One-way ANOVA, Tukey's post hoc; ***$p < 0.001$ saline vs. all other groups; $^{\dagger}p < 0.05$ sham controls vs. all other groups.

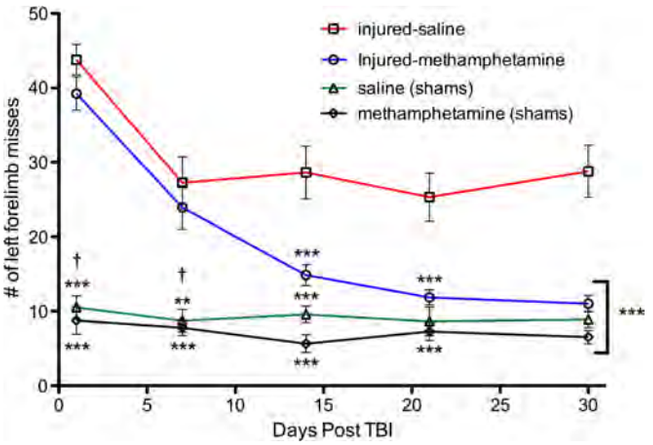

Figure 3. Low-dose methamphetamine treatment significantly improved foot fault assessments. Foot fault values are presented for injured methamphetamine-treated animals (*blue line*), injured saline-treated animals (*red line*), methamphetamine-treated sham controls (*black line*), and saline-treated sham controls (*green line*). Foot fault assessments were conducted weekly for up to 30 days; n = 13 for injured saline- and methamphetamine-treated animals, n = 8 for sham controls. One-way ANOVA, Tukey's post hoc; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ saline vs. groups, $^{\dagger}p < 0.05$ sham controls vs. groups.

showed significant improvement in the foot fault assessment when compared with saline-treated controls. By day 14, methamphetamine-treated animals were not significantly different from uninjured sham controls, and this effect continued for the remainder of the study (Fig. 3).

Low-Dose Methamphetamine Improves Cognitive Function After Severe TBI

Cognitive impairment is the most common deficit associated with TBI.[11] We used the MWM to determine the potential of methamphetamine to limit cognitive impairment after severe TBI. Animals were treated with methamphetamine or saline beginning 3 hours after injury. Training in the MWM began 3 weeks after injury to assess the effect of methamphetamine treatment on learning. On the first day of training, uninjured controls and injured methamphetamine- and saline-treated animals did not differ in the time required to locate the escape platform (latency, Fig. 4, *B*). However, by the third day of training, there were no statistically significant differences between the uninjured control rats and the injured rats that had been treated with methamphetamine. In contrast, injured rats that had been treated with saline had significantly longer latency times. Interestingly, methamphetamine-treated uninjured animals had significantly shorter latency times than all other groups on the first day of training. By day 3, there were no significant differences between methamphetamine-treated injured and uninjured sham controls.

A probe trial was conducted on day 6 to measure memory. For the probe trial, the escape platform was removed, and the time each animal spent searching for the platform in the target quadrant (quadrant 1 where the escape platform had been located) was used as a measure of functional memory. In addition, we determined the percent of time spent by each animal in the

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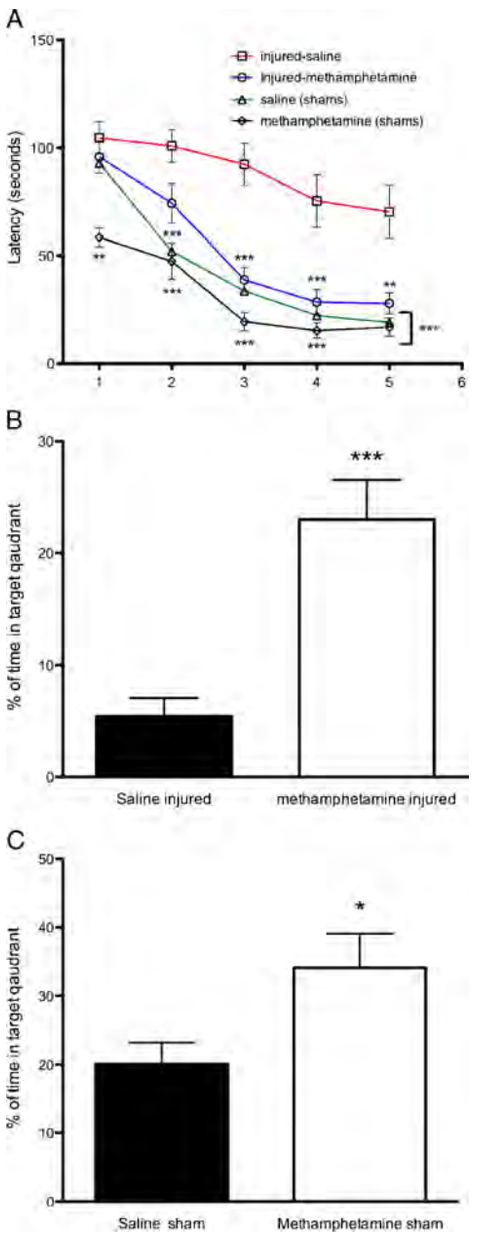

Figure 4. Low-dose methamphetamine treatment significantly improves learning and memory after TBI. Rats were trained in the MWM four times a day for 5 days beginning 24 days after njury to assess learning ability. (*A*) The average times required for rats to locate the escape platform (latency) are presented for each training day for injured methamphetamine-treated animals (*blue line*), injured saline-treated animals (*red line*), saline sham animals (*green line*), and methamphetamine-treated sham controls (*black line*); n = 8 for all groups. One-way ANOVA, Tukey's post hoc; $^{**}p < 0.01$ saline vs. all other groups, $^{***}p < 0.001$ saline vs. all other groups. Memory function was assessed in probe trials after training was completed. (*B*) Animals with TBI treated with saline or methamphetamine. (*C*) Injured animals that underwentsham surgeries and were treated with saline or methamphetamine. n = 7, two-tailed *t* test. $^{*}p < 0.05$; $^{***}p < 0.001$.

thigmotaxis zone (the area immediately adjacent to the edge of the tank). Injured methamphetamine-treated animals spent significantly more time (23%) searching in the target quadrant when compared with injured saline-treated rats (6%) (Fig. 4, *C*). In addition, the methamphetamine-treated animals spent significantly more time in the platform zone of quadrant 1 (the area where the escape platform had been located). Furthermore, the injured methamphetamine-treated animals spent significantly less time in the thigmotaxis zone (Fig. 4, *D*). As a control, we further examined the effects of methamphetamine treatment on cognitive function in uninjured rats. Methamphetamine-treated uninjured sham controls did not spend significantly more time searching in the target quadrant compared with uninjured saline-treated controls. However, methamphetamine-treated animals did spend significantly more time in quadrants 2 and 3 and within the platform zone and significantly less time in the thigmotaxis zone (Fig. 4, *E* and *F*). These data suggest that low-dose methamphetamine treatment enhanced learning and memory within the context of MWM testing.

Given the relatively long period between injury or treatment and cognitive assessment in the MWM (>3 weeks), we were interested in determining how methamphetamine prevented long-term cognitive impairment. The dentate gyrus region of the hippocampus contains a population of self-perpetuating neural stem cells. These cells differentiate into mature neurons that ultimately play a major role in learning and memory. As immature neurons, these cells express DCX. Therefore, we elected to determine if methamphetamine treatment affected the presence of immature DCX^+ neurons in the hippocampus. Paraffin-embedded brain sections were prepared at 48 hours after injury and treatment then stained for DCX expression. A significant increase in DCX^+ staining was observed in the dentate gyrus region of methamphetamine-treated animals compared with saline-treated controls (Fig. 5, *A* and *B*). Specifically, DCX^+ cells within the hippocampus of the injured hemisphere of methamphetamine-treated animals appeared more numerous and possessed more extensive dendritic processes than those of saline-treated animals. Interestingly, a similar increase in DCX^+ neurons with enhanced morphology was also observed in the dentate gyrus of the uninjured hemisphere of methamphetamine-treated animals compared with saline controls.

DISCUSSION

Most methamphetamine-based studies have used high, repetitive doses and have thus focused on the neurotoxic potential of the drug. These previous studies more closely modeled conditions associated with drug abuse.[5,6] At drastically reduced dosages, methamphetamine may have potential as a therapeutic. Currently, the drug is FDA approved, under the trade name Desoxyn, to treat attention deficit hyperactivity disorder in children. Interestingly, a recent retrospective 5-year study found that TBI patients testing positive for methamphetamine at the time of admission to the emergency room had a significant decrease in mortality.[12] The data we present here clearly demonstrates the neuroprotective potential of low-dose methamphetamine to improve both behavioral and cognitive function when administered 3 hours after severe TBI. These data indicate that methamphetamine treatment is protective after TBI. This conclusion is further

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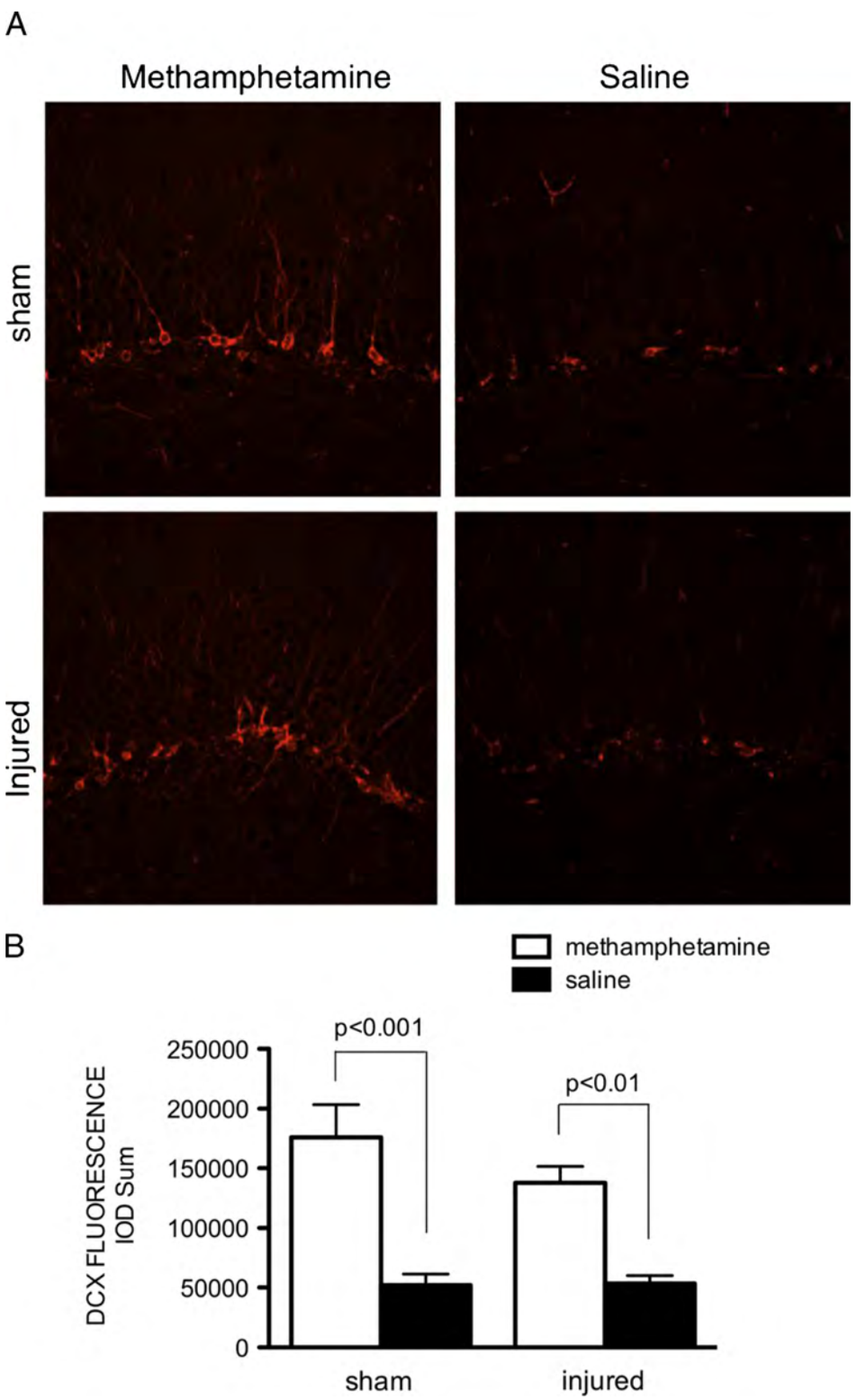

Figure 5. Low-dose methamphetamine treatment increases DCX staining in the dentate gyrus. Paraffin-embedded sections were prepared at 48 hours after injury and stained with anti-DCX antibody (red fluorescence). (*A*) Representative images from the injured and uninjured hemisphere of methamphetamine- and saline-treated animals. (*B*) DCX staining in each treatment group was quantified as total fluorescence intensity and presented as integrated optical density SUM. n = 4 with 17 replicates per group, statistical significance was determined by two-tailed *t* test.

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supported by the significant reduction in apoptotic cell death we observed in methamphetamine-treated animals. The delayed improvement we observed in behavioral function after methamphetamine treatment is particularly noteworthy. Twenty-four hours after injury, saline- and methamphetamine-treated animals did not differ significantly in behavioral assessments. However, 1 week after injury, the methamphetamine-treated animals showed a significant improvement in NSS and 2 weeks later showed a significant improvement in foot fault assessments. By 30 days after injury, the methamphetamine-treated animals matched the cognitive and behavioral performance of uninjured sham controls. Therefore, low-dose methamphetamine may not only promote protection but also exert a restorative effect.

The finding that methamphetamine treatment enhanced the presence and quality of DCX^+ immature neurons within the dentate gyrus suggests two possibilities. First, methamphetamine may preserve or promote survival of immature neurons already present in the subgranular zone of the hippocampus at the time of injury and treatment. Alternatively, methamphetamine treatment may enhance neurogenesis within this specialized population of neurons. The latter possibility seems more likely based on the observation that the level of DCX staining was independent of injury. Equivalent levels were seen in both the injured and the uninjured hemispheres of saline-treated animals. In contrast, enhanced DCX staining and preserved cell morphology was observed in both the injured and the uninjured hemispheres of methamphetamine-treated rats. These data suggest that methamphetamine promotes neurogenesis within this population of neurons, which may account for the enhanced performance of methamphetamine-treated animals observed in the MWM testing.

The molecular mechanisms of methamphetamine-mediated neuroprotection may be linked to dopamine release. Methamphetamine induces the release of dopamine and prevents subsequent reuptake and metabolism leading to increased dopamine receptor activation. Previous studies have elucidated several potential neuroprotective mechanisms involving dopamine receptor activation. It has been suggested that D_1 dopamine receptors elicit a neuroprotective response through direct interaction with the *N*-methyl D-aspartate receptor).[13] The activation of the D_1 dopamine receptor reduces Ca^{2+} currents through the *N*-methyl D-aspartate receptor, decreases excitotoxicity, and activates PI3 kinase (PI3K). It has been further suggested that the activation of D_2 dopamine receptors leads to an increase in PI3K activation and a reduction in AMPA receptors at the cell surface.[14] In addition, the activation of D_2 dopamine receptors has been shown to protect rat cortical neurons from glutamate excitotoxicity by activating antiapoptotic signaling through AKT and an up-regulation of Bcl-2 expression.[15] Dopamine may also reduce excitotoxicity through an AKT-mediated phosphorylation of the β2 subunit of the γ-aminobutyric acid A receptor, which results in an increased density of γ-aminobutyric acid A receptors within the postsynaptic membrane.[16,17]

Our previous data indicate that methamphetamine-mediated neuroprotection involves the activation of a dopamine–PI3K–AKT signaling pathway.[4] AKT is a critical, prosurvival kinase that suppresses several apoptotic mechanisms leading to neuronal survival.[18,19] Previous studies involving acute neuronal injury have shown that pAKT suppresses the activation of cleaved caspase 9 in neurons and inactivates proapoptotic Bcl-2-associated death promoter.[20,21] Furthermore, AKT phosphorylation of fork head at Thr24, Ser256, and Ser319 resulted in the nuclear export and inhibition of transcription factors leading to cell survival.[22,23] Taken together, these observations suggest that methamphetamine may produce a neuroprotective effect through the activation of multiple pathways.

SUMMARY

In the current study, we present evidence that low-dose methamphetamine, delivered 3 hours after a severe TBI, significantly improves behavioral and cognitive function and prevents apoptotic cell death. We further demonstrated that methamphetamine treatment increased the number and quality of differentiating, immature neurons in the dentate gyrus. Additional studies are currently underway to further define the therapeutic window, to establish the lowest effective dose, and to characterize the pharmacokinetics of the therapeutically effective dose.

AUTHORSHIP

D.J.P and T.F.R. contributed intellectually and technically to the design of the experiments, the analysis of all data, and the preparation of the article. A.S.K. and A.R.R. performed technical assistance with the generation of TBIs and behavioral assessments. A.S.K. performed all MWM testing. D.M.B. performed all histological experiments.

DISCLOSURE

T.F.R. and D.J.P. are listed as inventors on a patent application that is based on the technology presented in this article. The patent is owned by the University of Montana and has been licensed to Sinapis Pharma. D.J.P. serves as chief scientific officer for Sinapis Pharma. T.F.R. serves as a consultant for Sinapis Pharma. For the remaining authors, no conflicts of interest are declared.This study was supported by grant nos. CDMRP DR080702 (W81XWH-11-2-0026) and MBRCT 11-21.

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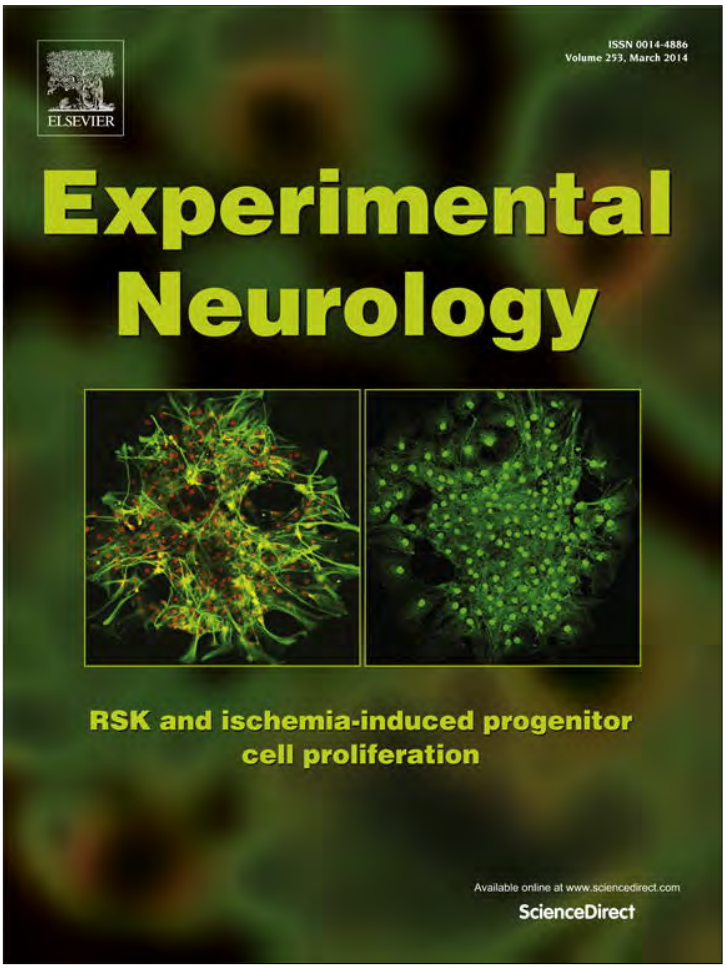

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journal homepage: www.elsevier.com/locate/yexnr

Administration of low dose methamphetamine 12 h after a severe traumatic brain injury prevents neurological dysfunction and cognitive impairment in rats ☆

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ARTICLE INFO

Article history:
Received 2 July 2013
Revised 20 November 2013
Accepted 2 December 2013
Available online 11 December 2013

Keywords:
Traumatic brain injury
Neuroprotection
Methamphetamine

ABSTRACT

We recently published data that showed low dose of methamphetamine is neuroprotective when delivered 3 h after a severe traumatic brain injury (TBI). In the current study, we further characterized the neuroprotective potential of methamphetamine by determining the lowest effective dose, maximum therapeutic window, pharmacokinetic profile and gene expression changes associated with treatment. Graded doses of methamphetamine were administered to rats beginning 8 h after severe TBI. We assessed neuroprotection based on neurological severity scores, foot fault assessments, cognitive performance in the Morris water maze, and histopathology. We defined 0.250 mg/kg/h as the lowest effective dose and treatment at 12 h as the therapeutic window following severe TBI. We examined gene expression changes following TBI and methamphetamine treatment to further define the potential molecular mechanisms of neuroprotection and determined that methamphetamine significantly reduced the expression of key pro-inflammatory signals. Pharmacokinetic analysis revealed that a 24-hour intravenous infusion of methamphetamine at a dose of 0.500 mg/kg/h produced a plasma C_{max} value of 25.9 ng/ml and a total exposure of 544 ng/ml over a 32 hour time frame. This represents almost half the 24-hour total exposure predicted for a daily oral dose of 25 mg in a 70 kg adult human. Thus, we have demonstrated that methamphetamine is neuroprotective when delivered up to 12 h after injury at doses that are compatible with current FDA approved levels.

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Introduction

Currently there are no approved therapeutic interventions available to prevent cognitive and behavioral deficits following traumatic brain injury (TBI) (Beauchamp et al., 2008). The development of viable treatments has been hindered by the fact that TBI represents a heterogeneous injury that activates multiple neuropathological pathways (Beauchamp et al., 2008; Dolan et al., 2012; Elder et al., 2010; Martin et al., 2008; Okie, 2005). During the primary injury phase, there is a rapid, uncontrolled release of glutamate that leads to calcium dysregulation, the production of reactive oxygen species (ROS), reactive nitrogen species (RNS), lipid peroxidation products, the release of prostaglandins, and the generation of nitric oxide (NO) (Pitkanen et al., 2009; Schober et al., 2012; Shultz et al., 2012; Stoica and Faden, 2010; Zhang et al., 2005). Consequently, these molecules induce microglial activation (Blaylock and Maroon, 2011; Block et al., 2007; Brown and Neher, 2010). Microglia are key mediators of both inflammatory responses and glutamate release (Brown and Neher, 2010). When activated in the presence of excessive glutamate, microglia exhibit a neuro-destructive phenotype and secrete pro-inflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-alpha), and interleukin-1 (IL-1) (Block et al., 2007; Brown and Neher, 2010; Stoica and Faden, 2010). The release of these cytokines induces a cascade of neuro-inflammation that perpetuates glutamate excitotoxicity and microglial activation eventually leading to neuronal loss and permanent neurological dysfunction (Blaylock and Maroon, 2011; Stoica and Faden, 2010).

Thus, survivors of severe TBI are likely to experience a degree of lasting neurological impairment, a permanent reduction in cognitive abilities, and psychological disturbances (Daneshvar et al., 2011; Dempsey et al., 2009). In the United States, 1.7 million individuals suffer from a TBI every year (Prevention C.f.D.C.a, 2006; Reeves and Panguluri, 2011; Saulle and Greenwald, 2012). Clearly, there is a crucial unmet need to develop novel, effective therapies that can be administered

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http://dx.doi.org/10.1016/j.expneurol.2013.12.001

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within a clinically relevant therapeutic window following TBI. We have recently published data demonstrating that administration of low dose methamphetamine following severe TBI resulted in improved functional and cognitive outcomes (Rau et al., 2012). In this previous study, we administered methamphetamine beginning 3 h after a severe TBI. While promising, a 3-hour delay to treatment does not represent a clinically relevant time frame. Therefore, in the present study we examined the therapeutic potential of methamphetamine at later time points. In addition, we more thoroughly characterized the pharmacology of methamphetamine by performing a dose response study and pharmacokinetic analysis. Finally, we examined critical gene expression changes in an effort to understand the potential molecular mechanisms of methamphetamine-mediated neuroprotection.

Methods

The Institutional Animal Care and Use Committee at The University of Montana approved all procedures in these studies. Adult, male, Wistar rats (350–500 g) were obtained from Charles River Laboratories (Wilmington, MA) and housed in filtered isolator boxes with a 12-hour light/dark schedule and ad libitum access to food and water. The lateral fluid percussion injury procedure was performed as we have previously published (Rau et al., 2012). Briefly, animals were deeply anesthetized using 2–4% isoflourane. A 5 mm trephination was made over the right hemisphere equidistant from the lambda and the bregma as we previously described (Rau et al., 2012). Animals were given a 20 ms fluid pulse to the dura at 1.9–2.3 ATM of pressure. Approximately 25% mortality was observed with these pressures. All animals became apneic after injury and were manually ventilated with supplemented O_2 until normal breathing resumed. At 7.5 or 11.5 h post-injury, an Alzet osmotic pump (Alzet corp; 2001D; 8.4 μL per hour) containing a methamphetamine solution designed to deliver 0.500, 0.250, or 0.250 mg/kg/h for 24 h was surgically implanted into the inguinal crease and connected to the femoral vein through a catheter as we previously described (Rau et al., 2012). The catheter (Scientific Commodities; Lake Havasu, AZ) from the pump was pre-loaded with a solution of 50% dextrose/50% heparin to prevent clotting. The length of the catheter and inner diameter were calculated to ensure methamphetamine delivery to the end of the catheter 30 min after insertion into the animal. At 8 or 12 h post injury, the animals were lightly anesthetized and a bolus dose of saline or methamphetamine was injected into the tail vein to coincide with the beginning of the pump delivery into the femoral vein. Bolus dosing for the 0.500 mg/kg/h dose was 0.425 mg/kg, 0.212 mg/kg for the 0.250 mg/kg/h dose, and 0.106 mg/kg for the 0.125 mg/kg/h dose. Saline and sham treated animals underwent the same procedure receiving pre-warmed saline. 72 h after injury the animals were re-anesthetized and the Alzet pumps were removed (Rau et al., 2012).

Neurological severity scoring

Neurological severity scoring (NSS) was performed as previously described (Rau et al., 2011, 2012). NSS and foot fault assessments were conducted on days 1, 7, 14, 21, 30, and day 40. Animals were scored from 0–16 with 16 indicating maximal impairment. Scoring criteria for a severe TBI was 16-10, Animals scoring 9 or less on day 1 were excluded as a moderate/mild injury.

Foot faults assessments

Foot fault assessments were conducted as previously described (Rau et al., 2012). Briefly, rats were set on an elevated grid. With each weight-bearing step, the paw may fall or slip off the wire grid. Each time the left forelimb (affected by damage to the right hemisphere) missed a placement on the wire rack it was recorded as a foot fault. The total number of steps (weight bearing movement of the right forelimb) that the rat used to cross the grid was counted, and the total number of foot faults for each forelimb was recorded.

Assessment of cognitive function

The Morris water maze (MWM) was used to assess the impact of methamphetamine on cognitive function (learning and memory) following TBI. The assessment procedure was performed as previously published (Rau et al., 2012). Pre-acclimation began on day 39 post-injury. The training phase began on day 40 post-injury, and the probe trial was conducted on day 45 post-injury. The water temperature was maintained at a constant 19 °Celsius with the clear plexiglas platform 2 cm below the water level. All data was recorded and analyzed using Anymaze software connected to a Logitech camera. All data sets were analyzed by a blinded researcher. There were no significant differences in swim speeds between any of the animals.

Immunohistochemistry

Forty-six days after TBI, rats were deeply anesthetized and perfused with 4% paraformaldehyde (PFA) fixative. Brains were post-fixed for 24 h at 4 °C in 4% PFA and divided into 2 mm coronal sections using a rat brain matrix. Slices were processed, paraffin embedded and sectioned at 7 μm. A 1 in 10 series from each rat was surveyed to identify location relative to Bregma. Sections were mounted at Bregma −3.3, deparaffinized and rehydrated. Antigen retrieval by treatment in 0.1 M citric acid pH 6 was performed, and sections were stained for a pan-axonal neurofilament marker (NF312 Covance, Princeton NJ) at a dilution of 1:1500 overnight at 4 °C in a humid chamber. Slides were washed with PBS 3 × 5 min and incubated with AlexaFluor 488 Goat anti Mouse IgG (Invitrogen, Life Technologies, Grand Island, NY) at a dilution of 1:300 for 1 h at room temperature in the dark. After rinsing in PBS 3 × 5 min nuclei were counterstained with DAPI (Invitrogen) diluted to 1 μg/ml in PBS for 5 min. Sections were rinsed in PBS, rinsed briefly in ddH_2O and coverslipped using FluorSave mounting media (Calbiochem, Darmstadt, Germany). Adjacent sections at Bregma −3.3 were deparaffinized, rehydrated, and permeabilized in 0.1% TritonX100 in PBS for 20 min. Following a PBS rinse slides were stained for 488 NeuroTrace (Molecular Probes, Life Technologies, Grand Island, NY) at a dilution of 1:400 overnight at 4 °C to label neurons. After NeuroTrace labeling, sections were rinsed in PBS 3 × 5 min and counterstained with DAPI as above. All sections were imaged on an Olympus Fluoview FV1000 confocal microscope (Olympus, USA), capturing the single brightest plane of fluorescence. For NF312 stained slides, images were captured at 40× (1.5 zoom) for the CA3 region of the hippocampus. Integrated Optical Density (IOD, a measure of area multiplied by the average density of staining) and axon length (a measure of total length of all one pixel thick open branches) of NF312 staining was measured on the injured hemisphere of the brain sections using ImagePro 6.2 software (ImagePro, Bethesda MD). The sum of the IOD or sum axon length from five saline, six 8 h and four 12 hour methamphetamine treated rats were measured. Treatment group averages of sum IOD or axon length and standard errors of the mean were calculated. Positive staining levels were identified by setting threshold levels from control slides reacted with secondary but no primary antibody. For the NeuroTrace stained slides images were captured at 20× (1.5zoom) in the CA1 region of hippocampus. Two sections per rat were imaged and dead cells were counted manually using ImagePro software. Live cells were round and contained NeuroTrace labeled (green) cytoplasm and blue (DAPI labeled) nuclei. Dead cells were very bright cells with condensed cytoplasm and nuclei and altered morphology. Average dead cells per rat and an average number of dead cells per treatment group were calculated. Four saline and four 8-hour methamphetamine and five 12 hour methamphetamine rats were counted and group averages and standard errors of the mean calculated.

RNA isolation/gene array protocol

Three biological replicates were used for each experimental group and each replicate was run in triplicate. Total RNA was isolated from the rat ipsilateral cortex utilizing Trizol LS (Invitrogen) according to the manufacturer's protocol. To remove any contaminating chemicals

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that could affect downstream analysis, RNA was further purified using the Qiagen RNeasy MinElute Cleanup Kit according to the manufacturer's protocol. RNA was quantified using a Nano Drop spectrophotometer. RNA was considered acceptable if the 260/280 and 260/230 ratios were 2.0 or greater. A 1 μg aliquot of total RNA was reverse transcribed to cDNA using Qiagen's RT2 First Strand Kit. One half of the cDNA reaction was mixed with 675 μl of Qiagen's 2X RT SYBR Green Mastermix and 624 μl H2O. This was then added to a custom array panel (SA Biosciences) and cycled according to the manufacturer's suggested qPCR protocol on a Bio-Rad iQ5 thermocycler. Values were normalized for GAPDH. For data analysis, the ΔΔCt method was used with the aid of the manufacturer's online software suite RT2 Profiler PCR Array Data Analysis version 3.5. Fold-changes were calculated and results compared to normal controls. Please note: (2^(- Delta Delta Ct)) is the normalized gene expression (2^(- Delta Ct)) in the test sample divided the normalized gene expression (2^(- Delta Ct)) in the Control Sample. Fold-regulation represents fold-change results in a biologically meaningful way. Fold-change values greater than one indicate a positive- or an up-regulation, and the fold-regulation is equal to the fold-change. Controls for this experiment underwent the trephination surgery but did not receive a TBI.

Measurement of methamphetamine from plasma

Jugular catheterized adult male Wistar rats were purchased from Charles River Laboratories. Rats were implanted with an Alzet pump (Alzet Corp., 2001D, 8.4 μl per hour) and a femoral vein catheter as previously described (Rau et al., 2012). Pump delivery was calculated based on pump rate and volume contained within the catheter. Based on the catheter inner diameter (ID) of 0.635 and a pump rate of 8.4 μl per hour +/−0.2 μl a catheter length of 2.25 cm delivered methamphetamine to the femoral vein at 1 h post-implantation. A total of six blood draws (500 μl each) were performed on each animal. Animals were anesthetized using 1–3% isoflurane and maintained at a plane of light sedation throughout the blood draw. Collected blood tubes were placed on ice and centrifuged immediately. Samples were spun at 1300 ×*g* for 10 min. Supernatants were immediately pulled off and placed in Eppendorf tubes. Samples were immediately frozen at −80 °C until processing and testing. Plasma samples were processed using the State of Montana crime lab extraction protocol and analyzed using LC-MS at the Missoula, Montana laboratory (Cravey, 1982; S. B., 1996).

Statistical analysis

All data was analyzed using Prizm software. To determine Gaussian (normal) distribution a Kolmogorof–Smirnov test was performed on all data sets. Appropriate parametric analysis was performed on data sets containing two groups using an unpaired, one-tailed *T*-test (CI = 95%). Analysis on data sets with more than 2 groups was done using one-way ANOVA with Tukey's post-hoc to determine statistical significance between groups. A $p < 0.05$ was considered significant.

Results

Methamphetamine improves behavioral outcomes when administration is delayed until 8 h after injury

We previously demonstrated that methamphetamine provides robust improvement in both behavioral and cognitive function when administration occurs within 3 h after severe TBI. However, treatment within 3 h is not clinically achievable in many cases. Therefore, we further characterized the neuroprotective potential and pharmacological profile of methamphetamine by conducting a dose response study with administration being delayed until 8 h after injury. Rats received a severe TBI and, 8 h later, were given an i.v. bolus injection of saline or methamphetamine (0.42, 0.21, or 0.10 mg/kg), followed by i.v. infusion for 24 h with saline or methamphetamine (0.50, 0.25, or 0.12 mg/kg/h), respectively. NSS and foot fault assessments were conducted on days 1, 7, 14, 21, 30 and 40 post-injury (Rau et al., 2012). Administration of methamphetamine at the 0.500 mg/kg/h or 0.250 mg/kg/h dose produced significant improvements in NSS on days 14, 21, 30 and 40 post injury (Fig. 1A). However, a significant improvement in neurological scoring for animals treated with 0.500 mg/kg/h dose was observed as early as 7 days post-injury, suggesting that the higher dose may have a slightly greater effect on early recovery from the injury. Animals receiving the 0.125 mg/kg/h dose had a significant improvement on day 14 post-injury however this effect did not persist. On days 1, 7, 21, 30 and 40, post-injury NSS for the 0.125 mg/kg/h treatment group did not differ from saline treated animals.

Dexterity, fine motor control and forepaw sensation was assessed using the foot fault test (Rau et al., 2012). Animals were examined on days 1, 7, 14, 21, 30, and 40 post-injury. Unlike the NSS assessment, all doses of methamphetamine improved performance in the foot fault assessment by day 14 post-injury (Fig. 1B). However, as with the NSS scoring, the 0.500 mg/kg/h dose showed improvement earlier (at 7 days post-injury) than the other doses did.

Methamphetamine improves cognitive outcomes when administration is delayed until 8 h after injury

The dose response effect of methamphetamine on cognitive performance following severe TBI was assessed using the Morris water maze (MWM). Dosing rats with 0.500 mg/kg/h or 0.250 mg/kg/h beginning 8 h after injury significantly reduced the time required to locate the

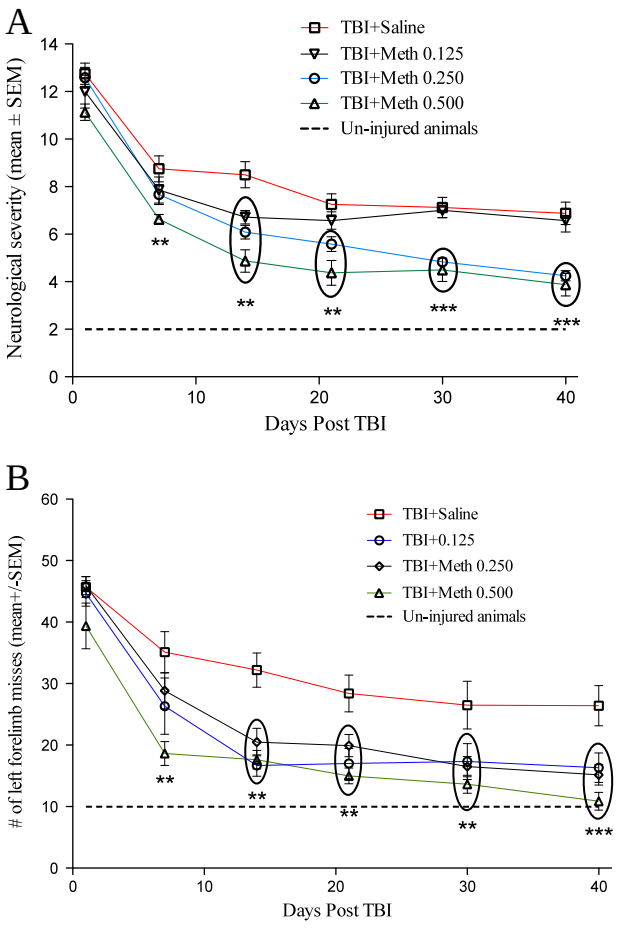

Fig. 1. Behavioral assessments following 8 hour delay of methamphetamine administration. Time course values are shown for (A) Neurological Severity Scores (NSS) and (B) foot fault assessments over 40 days for rats treated with saline (red line), 0.125 mg/kg/h (black line), 0.25 mg/kg/h (blue line) or 0.500 mg/kg/h (green line). The dashed line indicates typical NSS and foot fault scores for uninjured rats. n = a minimum of 8 animals per group. ** = $p < 0.01$, *** = $p < 0.001$ relative to saline treated controls.

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escape platform (Fig. 2A). A significant improvement in learning was observed on training days 3, 4, and 5 for rats treated with the 0.500 mg/kg/h dose. Rats treated with this dose behaved similar to uninjured rats by the third training day. However, rats treated with 0.250 mg/kg/h did not show significant improvement over saline treated controls until the fifth training day. In contrast, the 0.125 mg/kg/h treated rats showed no significant improvement over saline controls on any of the days tested indicating this dose failed to improve learning following severe TBI.

After completion of the five-day training period, we used a probe trial to assess memory function. Using this assessment of spatial memory, we found that injured rats treated with either 0.500 mg/kg/h or 0.250 mg/kg/h methamphetamine spent significantly greater time in the target quadrant searching for the escape platform (Fig. 2B) and significantly less time (~25%) in the thigmotaxia zone (Fig. 2C) compared to saline treated, injured animals. The thigmotaxia zone is the circular area around the outer rim of the tank and was not included in the four search quadrants. Animals that spend time in this zone are not actively searching or using visual cues and thus swim time spent in the thigmotaxia zone represents a random search pattern that does not involve active spatial memory recall. Animals treated with 0.125 mg/kg/h methamphetamine did not differ from saline treated, injured animals in the amount of time they spent in the target quadrant or the thigmotaxia zone, indicating that the 0.125 mg/kg/h dose of methamphetamine had no effect on memory improvement after TBI.

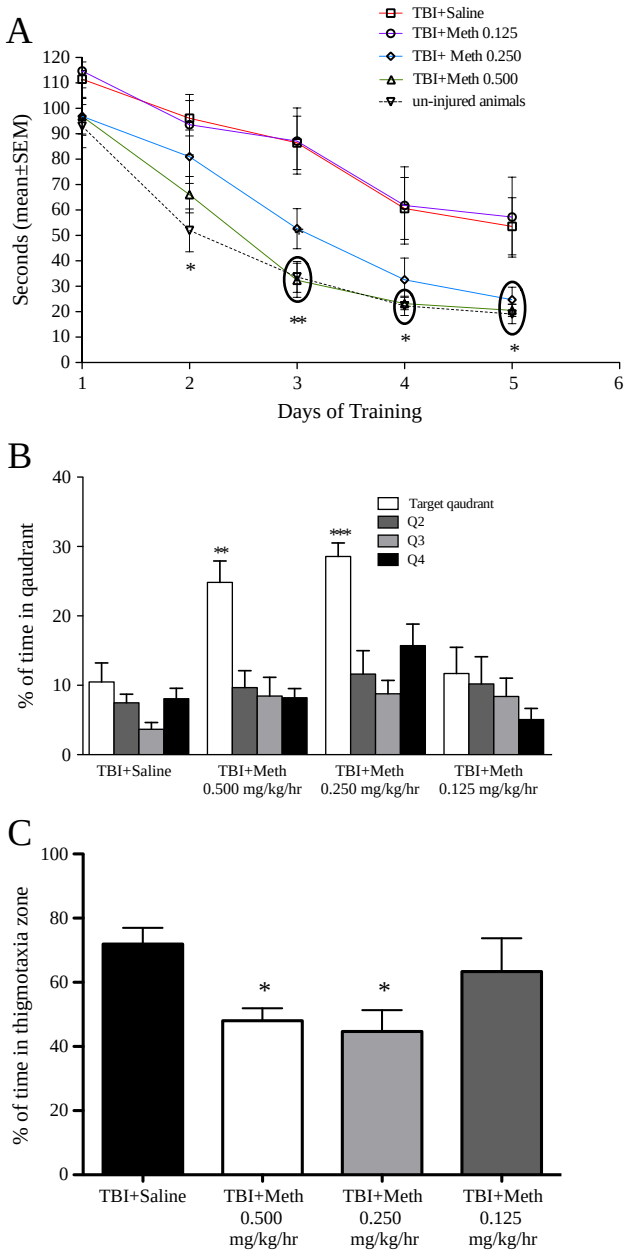

Fig. 2. Treatment with methamphetamine improves learning and memory after severe TBI. (A) Shows latency times over 5 training days for rats treated with saline (red line), 0.125 mg/kg/h (black line), 0.250 mg/kg/h (blue line) or 0.500 mg/kg/h (green line) beginning 8 h after severe TBI. The dotted black line indicates the latency times for uninjured control rats. (B) Shows the percent time rats spent in each quadrant of the maze searching for the escape platform. The target quadrant (white bars) is where the escape platform had previously been located. Non-target quadrants were Q2-4. (C) Shows the percent of time that rats from each treatment group spent within the thigomataxia zone. n = 8 animals per group. * = $p < 0.05$, ** = $p < 0.01$ relative to saline treated controls.

Methamphetamine is neuroprotective when administration is delayed up to 12 h post TBI

To further characterize the pharmacology of methamphetamine as a potential neuroprotective agent and define possible parameters of clinical application, we examined the therapeutic window of treatment. To accomplish this, we delayed delivery of methamphetamine until 12 h after severe TBI. We again used NSS and foot fault tests to assess

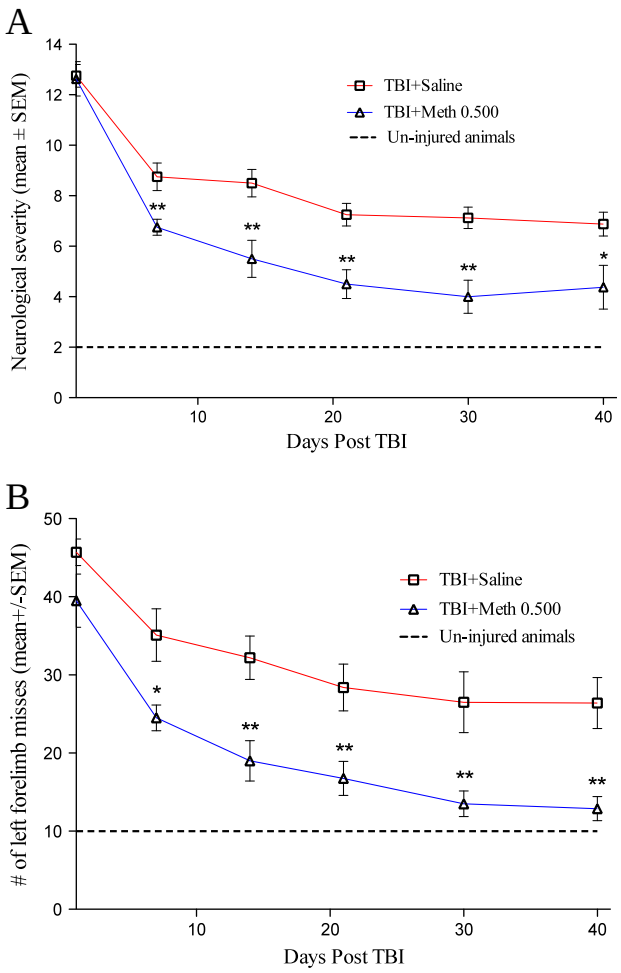

Fig. 3. Methamphetamine administered 12 h after TBI reduced neurological severity scores. Time course values are shown for (A) Neurological Severity Scores and (B) foot fault assessments over 40 days for rats treated with saline (red line), or 0.500 mg/kg/h (blue line). The dashed line indicates typical NSS and foot fault scores for uninjured rats. n = a minimum of 8 animals per group. * = $p < 0.05$, ** = $p < 0.01$, relative to saline treated controls.

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functional behavior. We observed a significant improvement in both NSS and foot fault measurements within 7 days following delayed administration (Fig. 3).

Spatial learning and reference memory were again assessed in the MWM (Rau et al., 2012). As with the NSS and foot fault testing, delaying administration of methamphetamine until 12 h after severe TBI resulted in significantly improved performance in the MWM. Similar to the 8-hour delivery study, we observed a robust improvement in memory and a significant reduction in the time spent in the thigmotaxia zone (Fig. 4). However, rats treated with methamphetamine beginning at 12 h after injury did not show a significant improvement in latency times until the fifth day of training. This represents a substantial shift in the learning curve relative to the 8-hour study and indicates that delaying methamphetamine treatment to 12 h post-TBI reduces improvement in this outcome.

Methamphetamine treatment reduces neuronal loss and preserves axon density after severe TBI

Immunofluorescent analysis was conducted on paraffin embedded brain sections from the injury area. Sections were prepared at 46 days after injury. As previously described by Ooigawa et al. (2006) we utilized a fluorescent Nissil stain (NeuroTRACE), to label both live and dead neurons in injured animals treated with saline or methamphetamine (Ooigawa et al., 2006). We found that treating rats with 0.500 mg/kg/h methamphetamine beginning at 8 or 12 h after severe TBI significantly reduced neuronal loss in the CA1 region of the hippocampus compared to injured, saline treated control animals (Fig. 5). Diffuse axonal injury is often observed following TBI. Therefore, we assessed the potential of methamphetamine to preserve axonal density. Using a pan-antibody for neurofilament, we found that delivering methamphetamine at the 0.500 mg/kg/h dose, beginning 8 h after injury, significantly preserved neurofilament staining within the CA3 region of the hippocampus when compared to saline treated control animals (Fig. 6). However, there was no significant difference in neurofilament staining in the hippocampus when treatment was delayed until 12 h post-injury.

Pharmacokinetics at a therapeutic dose of methamphetamine

To more fully characterize the pharmacology of methamphetamine we examined the pharmacokinetic profile of the highest therapeutically effective dose tested. A 24-hour infusion of methamphetamine at 0.500 mg/kg/h for 24 h generated a C_{max} of 25.9 ng/ml and an exposure of 17 ng/ml/h over a 32-hour period. This produced a total drug exposure of 544 ng/ml. The FDA recommended oral dose for treating ADHD is up to 25 mg daily (Administration F.a.D, 2007). Based on this dosing regimen, we calculated a 24-hour total drug exposure of 998 ng/ml, for a 70 kg human receiving a daily oral dose of 25 mg. We also analyzed the bioactive metabolite D-amphetamine, and found that exposure to 0.500 mg/kg/h methamphetamine generated a C_{max} of 13.3 ng/ml and a total drug exposure over 32 h of 252 ng/ml (Fig. 7).

Methamphetamine significantly alters the expression of genes involved in multiple pathophysiological pathways

We previously reported that methamphetamine mediates neuroprotection through dopamine-dependent activation of a PI3K-AKT signaling pathway (Rau et al., 2011). We have further demonstrated that methamphetamine significantly reduced apoptosis after severe stroke and TBI (Rau et al., 2011). In addition to the histological evidence of neuroprotection presented in this study, we wanted to further define the potential mechanisms of methamphetamine-mediated neuroprotection. Therefore, we conducted a gene array analysis to screen for candidate genes involved in the pathological processes induced by severe TBI. As Table 1 indicates, methamphetamine treatment prevented the increased expression of several key genes involved in inflammatory signaling. Many of the largest increases (when compared to sham, un-injured animals) we observed in gene expression involved pro-inflammatory molecules. For example, the chemokine CCL2 (11 fold increase, $p = 0.0004$), the Toll-like receptor signaling protein Myd88 (3 fold increase, $p < 0.0001$) and the pro-inflammatory cytokine IL1β (4.6 fold increase, $p = 0.005$) were all significantly elevated in saline treated TBI injured

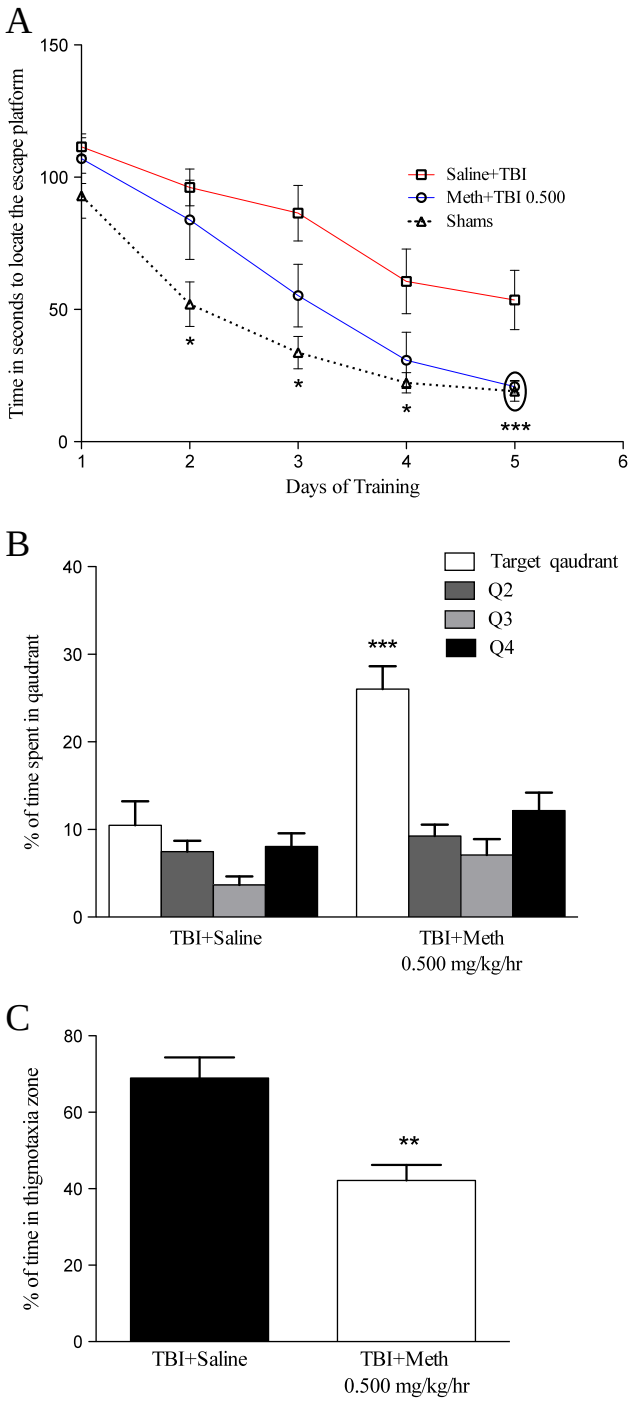

Fig. 4. Treatment with low dose methamphetamine improved learning and memory when delivered 12 h after a severe TBI. (A) Shows latency times over 5 training days for rats treated with saline (red line), or 0.500 mg/kg/h (blue line) beginning 12 h after severe TBI. The dotted black line indicates the latency times for uninjured control rats. (B) Shows the percent time rats spent in each quadrant of the maze searching for the escape platform. The target quadrant (white bars) is where the escape platform had previously been located. Non-target quadrants were Q2-4. (C) Shows the percent of time rats from each treatment group spent within the thigomataxia zone. n = 8 animals per group. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ relative to saline treated controls.

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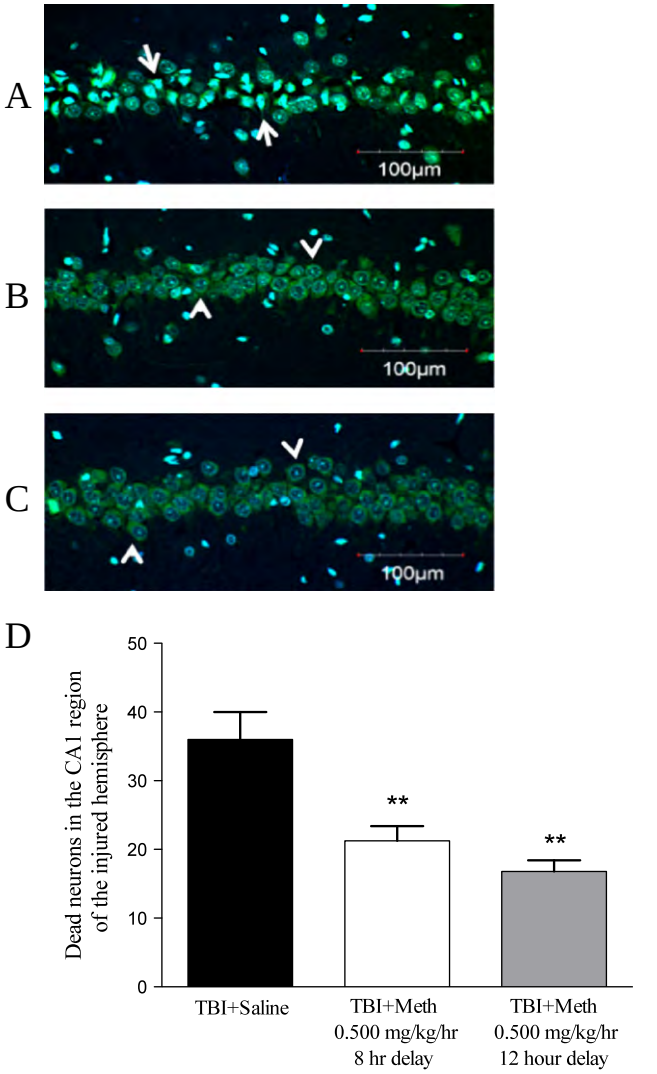

Fig. 5. Methamphetamine is neuroprotective in the CA1 region of the hippocampus at 46 days after severe TBI. Representative micrographs of the CA1 region at Bregma −3.3 show increased numbers of dead cells in saline treated TBI animals (A) compared with rats treated with 0.500 mg/kg/h methamphetamine at 8 h post TBI (B) or at 12 h post-TBI (C). Nuclei are stained blue with DAPI. Dead and dying neurons stain brightly green with NeuroTrace with little or no nuclei (arrows in a). Normal neurons have blue nuclei and rounded cell morphology with a large cytoplasm that stains less intensely green (arrowheads in b and c). (D) Shows graphically the average values for each treatment group. n = 4 animals per group. ** = $p < 0.01$.

animals. Conversely, we did not observe significant changes in the expression of CCL2, Myd88 or IL1β in the methamphetamine treated rats. However, methamphetamine treatment did induce a significant increase in the anti-inflammatory chemokine CXCL12 (2.2 fold, $p = 0.0001$) while saline controls showed no significant change. Finally, we observed at least a two-fold increase in the expression of corticotropin releasing hormone ($p = 0.0006$), and neurotensin ($p = 0.008$) in methamphetamine treated rats compared to a 1.7 fold decrease ($p = 0.002$) in corticotropin releasing hormone and no significant change in neurotensin in saline treated controls.

Discussion

In this study we have established 0.250 mg/kg/h as the lowest effective dose of methamphetamine that significantly reduces neurological dysfunction and cognitive impairment when delivered 8 h after a severe

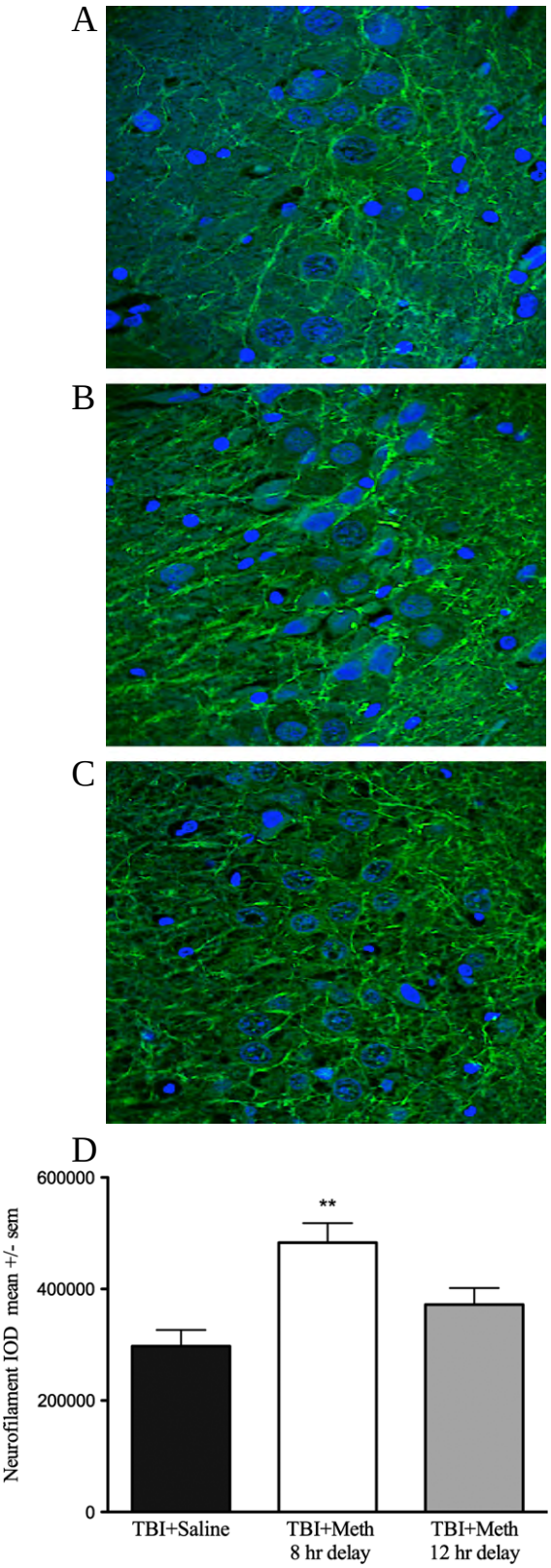

Fig. 6. Methamphetamine preserves axonal structure. Representative images of the CA3 region show decreased neurofilament staining in saline treated TBI injured animals (A) compared to TBI rats treated with methamphetamine at 8 h after injury (B). Animals treated with methamphetamine beginning 12 h post TBI did not show a significant difference from saline treated TBI animals (C). Quantification of neurofilament staining showed significantly greater axonal staining (d) in rats treated with methamphetamine beginning 8 h after injury, but not at 12 h, (D). n = minimum of 4 rats per group. * = $p < 0.05$, ** = $p < 0.01$.

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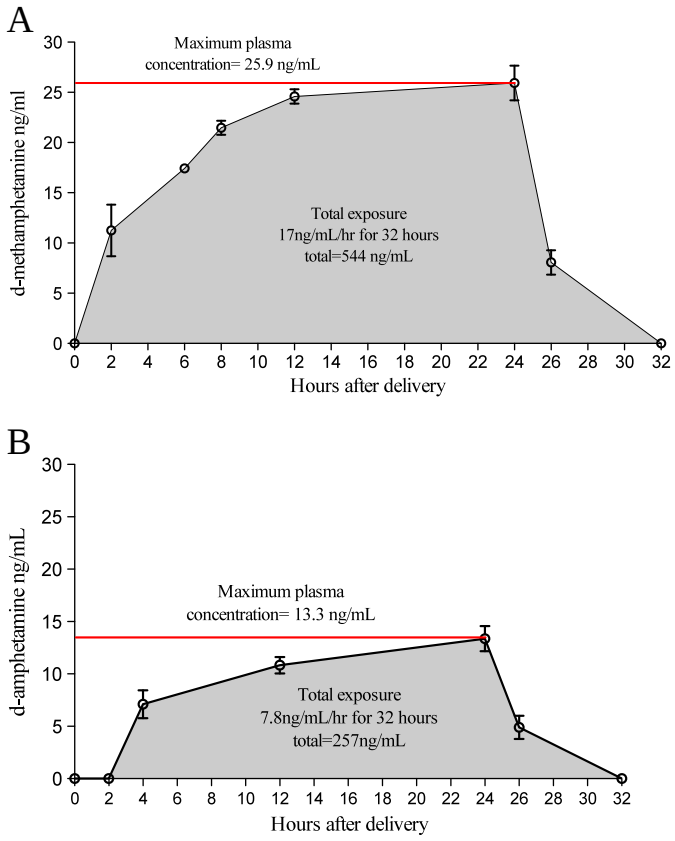

Fig. 7. Pharmacokinetics of D-methamphetamine and its metabolite, D-amphetamine. The concentrations of methamphetamine (A) and amphetamine (B) detected in the plasma of rats treated with methamphetamine at a dose of 0.500 mg/kg/h for 24 h are shown.

TBI. We further established that methamphetamine produced significant behavioral and cognitive improvements when a dose of 0.500 mg/kg/h is delivered up to 12 h after a severe TBI. Immediately after TBI, transient mitochondrial swelling occurs, which after 12 h, leads to irreversible diffuse axonal injury (Blaylock and Maroon, 2011; Saulle and Greenwald, 2012; Stoica and Faden, 2010; Thompson et al., 2005). Thus, the inability of methamphetamine to preserve axons after a 12-hour delay may directly correlate with these axonal studies. It may also explain why delaying methamphetamine treatment until 12 h after injury had less of an impact on the learning assessments. Despite the loss of neurofilament staining in the CA3 region, animals in the 12-hour delay group still demonstrated a significant improvement in memory. The disparity between the learning and memory outcomes may be explained by our observation that the 12-hour delay group still had a significant preservation of CA1 pyramidal neurons, which are critical in memory consolidation (Wittenberg et al., 2002). This suggests that low dose methamphetamine can be delivered up to 12 h after injury and still protect highly vulnerable neurons within the hippocampus.

Table 1
Gene expression analysis in TBI rats.

	Fold change	
Gene	Saline/normal	Methamphetamine/normal
CCL2	11 (0.0004)	NSC
MyD88	3 (0.0001)	NSC
IL1b	4.6 (0.005)	NSC
CXCL12	NSC	2.2 (0.0001)
CRH	−1.7 (0.002)	2 (0.0006)
neurotensin	NSC	2 (0.008)

NSC = no significant change. P values relative to normal (uninjured) rats are shown in parenthesis.

Of particular interest in this study was our finding that methamphetamine improved NSS scoring and foot faults but reduced cell death and axonal loss only in the hippocampus. A comprehensive literature search provides a spectrum of contradicting outcomes in the context of motor function relative to cortical injury in the rat LFP model. Selected LFP studies have shown a correlation between a reduction in cortical lesion volume and motor function improvement (Luo et al., 2013; Otani et al., 2007). In contrast, other studies have shown improved motor behavior with no decrease in cortical lesion volume (Lippert-Gruener et al., 2007). Further studies have reported neuroprotective treatments that improved lesion volume but did not improve motor function (Marklund et al., 2001).

Others have shown a correlation between reduced hippocampal damage following LFP-induced TBI and improved motor function (Lee et al., 2004). Similar to our studies, Lee et al. (2004) only reported pathological alterations in the hippocampus. Supporting this observation, Gheysen et al. (2010) presented evidence that the hippocampus contributes to early and late stage motor sequence learning that is critical to performance of motor function tasks (Gheysen et al., 2010). Further supporting the role of the hippocampus in motor tasks Grossberg and Merrill (1996), found that the hippocampus plays a key role in controlling adaptive timing and spatial orientation which are two key elements of balance and coordination, both of which are tested in the NSS scoring and foot fault analysis (Grossberg and Merrill, 1996; Gorchetchnikov and Grossberg, 2007). Specifically, Grossberg found clear evidence that the hippocampal circuits play a key role in cerebellar timing control, spatial orientation, and motor outputs (Grossberg and Merrill, 1996; Gorchetchnikov and Grossberg, 2007; Grossberg and Seidman, 2006).

The LFP model creates a global stress on the brain and generates the coup-contre coup effect found in closed head injuries (Xiong et al., 2012). Interestingly, closed head injuries can produce motor deficits but fail to generate a cortical lesion, (or measurable damage) within the motor cortex (Heitger et al., 2006). Heitger et al. (2006) also found that despite the lack of measureable tissue abnormalities, a mild closed head TBI could produce motor deficits up to 1 year following the injury (Heitger et al., 2006).

Another potential explanation may be linked to white matter track remodeling that occurs after TBI. We previously reported (Ding et al., 2013) that methamphetamine treatment improved foot faults and NSS scores but did not decrease cortical lesion volumes (Ding et al., 2013). However, we did observe a statistically significant increase in white matter track remodeling within the peri-lesional region of the cortex in methamphetamine treated animals that was not present in saline treated controls. Interestingly, this significant change was not observed until 5 and 6 weeks post injury suggesting methamphetamine treatment may have a long-term effect irrespective of the initial lesion core (Ding et al., 2013).

After administration, methamphetamine rapidly concentrates in the brain at significantly higher levels than in the plasma (up to 13 times greater) and induces the immediate release of dopamine, norepinephrine and serotonin (Administration F.a.D, 2007; Krasnova and Cadet, 2009; S. B., 1996). Methamphetamine also blocks the degradation of these neurotransmitters, resulting in prolonged neuronal signaling (Cadet and Krasnova, 2009; Krasnova and Cadet, 2009). When used at very high doses it produces profound euphoria, increased heart rate, respiration, agitation and dopamine mediated neurotoxicity (Cadet and Krasnova, 2009; Krasnova and Cadet, 2009). However, when administered at low doses, methamphetamine produces a more controlled release of catecholamines, capable of activating multiple neuroprotective pathways in the brain (Feeney and Hovda, 1983; Feeney et al., 1981, 1982; Rau et al., 2011, 2012). Currently, methamphetamine is FDA approved for the treatment of ADHD and obesity as an oral daily dose of up to 25 mg (Administration F.a.D, 2007). Under this dosing regimen, the total 24-hour exposure for a 70 kg adult would be twice what we

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observed with the highest therapeutic dose (0.500 mg/kg/h) tested in rats. However, recently published data indicates that a single i.v. injection of 0.5 mg/kg methamphetamine in a healthy human test subjects produced a 24 h total exposure similar to what a daily oral dose of 25 mg would produce (Mendelson et al., 2006). This suggests that the current FDA approved dose may more than sufficient to provide neuroprotective efficacy in humans.

A recent study by Vaarmann et al. (2013) suggested that dopamine prevents delayed calcium deregulation and glutamate excitotoxicity (Vaarmann et al., 2013). This is relevant as TBI produces glutamate-mediated excitotoxicity and calcium dysregulation. This effect was demonstrated to be dependent on the activation of D1 and D2 receptors and was enhanced by the addition of a monoamine oxidase inhibitor. We have previously reported that methamphetamine-mediated neuroprotection in a stroke model was dependent on dopamine activation of D1 and D2 receptors (Administration F.a.D, 2007). As a secondary effect, methamphetamine also inhibits monoamine oxidase activity. Thus, methamphetamine may be exerting a neuroprotective effect by preventing delayed calcium deregulation and excitotoxicity. Furthermore, we have also previously demonstrated that delivering low dose methamphetamine increased protein kinase B (AKT) phosphorylation in a dopamine dependent manner (Rau et al., 2011). This finding is also linked to neuroprotection as AKT is a central kinase that blocks multiple pathways of apoptotic death including BAD, Bim, GSK3β, p53 and Fox01 (Rau et al., 2011; Zhang et al., 2005). Further supporting the potential anti-apoptosis effect of AKT phosphorylation, we found that methamphetamine treatment significantly reduced apoptotic neuronal death in the hippocampus.

In addition to apoptosis, TBI induces a cascade of pathophysiology involving a broad spectrum of gene expression changes. We observed large changes in the expression of key genes that influence cerebral inflammation 32 h after TBI. Specifically, methamphetamine treatment significantly reduced cortical expression of three key mediators of inflammation: CCL2, MyD88 and IL1ß.

The pro-inflammatory chemokine CCL2 (MCP-1) increased 11 fold in injured, saline treated animals, whereas treatment with methamphetamine completely blocked this increase. A member of the monocyte chemotactic protein (MCP) family, CCL2 is expressed by astrocytes, macrophages, and reactive microglia, and is one of the most potent chemo-attractants that recruit monocytes to damaged areas (Sozzani et al., 1994a, 1994b). In addition, there is direct evidence that CCL2 contributes to the pathogenesis and development of brain lesions from ischemia and excitotoxic insults, both of which play a role in the development of pathology following TBI (Semple et al., 2010, 2011). Supporting this finding is the observation that CCL2 knockout mice have significantly lower neurological impairment following TBI relative to wild type mice (Lloyd et al., 2008). A rapid increase in CCL2 is observed within human spinal fluid following severe TBI and CCL2 levels remain significantly elevated over controls for up to 10 days post-injury (Semple et al., 2010). Semple et al. (2010) recently reported that increased levels of CCL2 were detected in the serum of patients who died shortly after TBI (Semple et al., 2010). Thus, a direct correlation exists between increased CCL2 levels, and increased neuro-inflammation and neuropathology following TBI. Our findings suggest that methamphetamine mediates neuroprotection in part, through the reduction of this critical neuro-inflammatory mediator.

The concept that methamphetamine treatment reduces post-traumatic inflammation is further supported by the fact that TBI induced a 3-fold increase in the expression of the myeloid differentiation primary response protein 88 (Myd88) gene, while a significant increase in Myd88 expression was absent in the methamphetamine treated rats. Myd88 is a key adaptor protein involved in Toll-like receptor and pro-inflammatory cytokine signal transduction. Activation of Toll-like receptors results in the recruitment of Myd88 and subsequent activation of the transcription activator, nuclear factor kappaB (NF-κB). NF-κB induces the rapid expression of pro-inflammatory molecules including tumor necrosis factor a (TNF-α), IL-1β, interleukin-6 (IL-6), and intracellular adhesion molecule-1 (ICAM-1) (G.Z. Li et al, 2011.; W. Li et al., 2013; Ling et al., 2013). This may explain the 4.6 fold increase we observed in IL-1β after TBI. Li et al. (2011) demonstrated a significant increase in Myd88 positive cells surrounding the TBI injury zone and a positive correlation between the expression of Myd88, NF-κB, and IL-1β (G.Z. Li et al., 2011; W. Li et al., 2013; Zhang et al., 2012). Further supporting the inflammatory role of Myd88 in TBI, Li et al. (2011) found that Myd88 was significantly elevated 72 h after TBI in humans and was actively expressed in neurons, astrocytes, and microglia (G.Z. Li et al., 2011; W. Li et al., 2013). This correlated with a significant increase in TNF-α and IL-1β (W. Li et al., 2013; G.Z. Li et al., 2011).

In our experiments, we observed Myd88, CCL-2, and IL-1β expression levels in methamphetamine treated TBI animals that were not significantly different from uninjured sham controls 32 h after TBI. The exact mechanism(s) of how methamphetamine produced the gene changes we observed are not known, and there are no current studies that link methamphetamine to decreased neuroinflammation. There are studies, however, that suggest a link between dopamine signaling and decreased inflammation. Shao et al. (2012) demonstrated that the astrocytic D2 dopamine receptor (D2R) potently suppressed neuroinflammation in the rodent brain by influencing expression of αB-crystallin (CRYAB) (Shao et al., 2012). Coffeen et al. (2010) demonstrated that brain inflammation resulted in a significant decrease in the release of dopamine, DOPAC, and HVA. Inflammation also decreased the mRNA levels of the D1R suggesting that inflammation negatively regulates dopamine signaling (Coffeen et al., 2010). In our previous studies of methamphetamine using a focal embolic stroke model we found that low dose methamphetamine exerted a neuroprotective effect that was dependent on a PI3K/AKT pathway. Interestingly, activation of the PI3K/AKT pathway, in addition to suppressing apoptotic factors, is necessary for activation of nuclear factor erythroid 2-related factor 2 antioxidant response element (Nrf2-ARE). Nrf2-ARE is commonly involved in the transcriptional regulation of genes encoding antioxidant proteins such as glutathione (GSH) under stress conditions (Chen et al., 2009; Jin et al., 2008). Thus, increasing the presence and activity of Nrf2-ARE through AKT would likely decrease ROS production after TBI, and there is a growing body of evidence indicating that ROS directly affects the expression of pro-inflammatory genes. Gupta et al. (2012) demonstrated that ROS activate various transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB], activator protein-1, hypoxia-inducible factor-1α, and signal transducer and activator of transcription 3, resulting in the expression of proteins that control inflammation, (Gupta et al., 2012). While we do not know if a methamphetamine-dopamine-PI3K/AKT pathway is activating Nrf2-ARE leading to decreased ROS, the potential neuroprotective pathway warrants future investigation. An Nrf2-ARE mediated decrease in ROS may be altering expression of the pro-inflammatory genes we observed in the cortex of methamphetamine treated TBI animals. Testing these hypotheses in future studies will hopefully elucidate the mechanism(s) by which methamphetamine reduces cognitive and neurological impairment after TBI.

We also observed additional changes in the gene expression profile that further suggest methamphetamine treatment impacts multiple pathophysiological pathways. For example, we detected a 2-fold increase in the expression of corticotropin-releasing hormone (CRH) in the brains of methamphetamine treated rats following TBI, compared to a 1.7 fold decrease in saline treated controls. CRH has been shown to protect primary cortical and hippocampal neurons from apoptosis when administered 8 h after an acute neurological insult (Stevens et al., 2003). The in-vivo administration of CRH after global ischemia in Wistar rats increased survival of CA1 neurons by 61% (Charron et al., 2008). Similar to our observations here, Charron et al. (2008) demonstrated that CRH treatment could be delayed until 8 h after ischemic injury and that treatment significantly improved spatial memory and preserved CA1 neuronal survival (Charron et al., 2008). Finally,

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Koutmani et al. (2013) recently demonstrated that CRH promotes the proliferation and survival of neuronal progenitor cells (Koutmani et al., 2013). This is noteworthy as we recently demonstrated that methamphetamine treated rats have significantly more doublecortin positive, immature neurons within the subgranular zone of the dentate gyrus after TBI compared to saline treated controls (Administration F.a.D, 2007). This suggests that methamphetamine may promote proliferation and survival of neuronal precursors through the activity of CRH.

Conclusion

The data presented in this study indicate that low dose methamphetamine is neuroprotective when delivered 8–12 h after a severe TBI in a rodent model. In addition to reducing neurological and cognitive impairment, methamphetamine reduced neuronal death in the hippocampus when delivered up to 12 h after TBI. These data, combined with our previously published studies, suggest a novel, neuroprotective use for methamphetamine.

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MRI of Neuronal Recovery after Low-Dose Methamphetamine Treatment of Traumatic Brain Injury in Rats

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Abstract

We assessed the effects of low dose methamphetamine treatment of traumatic brain injury (TBI) in rats by employing MRI, immunohistology, and neurological functional tests. Young male Wistar rats were subjected to TBI using the controlled cortical impact model. The treated rats (n = 10) received an intravenous (iv) bolus dose of 0.42 mg/kg of methamphetamine at eight hours after the TBI followed by continuous iv infusion for 24 hrs. The control rats (n = 10) received the same volume of saline using the same protocol. MRI scans, including T2-weighted imaging (T2WI) and diffusion tensor imaging (DTI), were performed one day prior to TBI, and at 1 and 3 days post TBI, and then weekly for 6 weeks. The lesion volumes of TBI damaged cerebral tissue were demarcated by elevated values in T_2 maps and were histologically identified by hematoxylin and eosin (H&E) staining. The fractional anisotropy (FA) values within regions-of-interest (ROI) were measured in FA maps deduced from DTI, and were directly compared with Bielschowsky's silver and Luxol fast blue (BLFB) immunohistological staining. No therapeutic effect on lesion volumes was detected during 6 weeks after TBI. However, treatment significantly increased FA values in the recovery ROI compared with the control group at 5 and 6 weeks after TBI. Myelinated axons histologically measured using BLFB were significantly increased ($p<0.001$) in the treated group (25.84±1.41%) compared with the control group (17.05±2.95%). Significant correlations were detected between FA and BLFB measures in the recovery ROI ($R = 0.54$, $p<0.02$). Methamphetamine treatment significantly reduced modified neurological severity scores from 2 to 6 weeks ($p<0.05$) and foot-fault errors from 3 days to 6 weeks ($p<0.05$) after TBI. Thus, the FA data suggest that methamphetamine treatment improves white matter reorganization from 5 to 6 weeks after TBI in rats compared with saline treatment, which may contribute to the observed functional recovery.

Citation: Ding GL, Chopp M, Poulsen DJ, Li L, Qu C, et al. (2013) MRI of Neuronal Recovery after Low-Dose Methamphetamine Treatment of Traumatic Brain Injury in Rats. PLoS ONE 8(4): e61241. doi:10.1371/journal.pone.0061241

Editor: Jiani Hu, Wayne State University, United States of America

Received January 3, 2013; **Accepted** March 6, 2013; **Published** April 18, 2013

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Funding: This work was financially supported by Department of Defense Grant CDMRP DR080702 (W81XWH-11-2-0026) and National Institutes of Health RO1 NS064134. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: DJP is listed as an inventor on a patent application that is based on the use of methamphetamine in the treatment of TBI. The patent is owned by the University of Montana and has been licensed to Sinapis Pharma. DJP serves as Chief Scientific Officer for Sinapis Pharma. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

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Introduction

Traumatic brain injury (TBI) is a leading factor of morbidity and mortality in Western countries [1,2], and an important public health problem. There is an urgent need to develop a novel approach for the treatment of TBI, that can restore brain function. TBI produces a primary insult, which may trigger widespread brain damage regardless of the original site of injury [3]. Thus, patients with TBI may suffer from axonal degeneration and generalized cerebral atrophy associated with poor neurological functions [4–8]. Unfortunately, the management of patients with TBI to-date has primarily focused on therapeutic intervention designed to reduce cellular damage and brain edema [9]; currently, no effective clinical treatment can repair biostructural damage to the neurons and prevent or reduce secondary pathological processes [10]. However, experimental pharmacological and cell-based treatments of TBI which promote brain remodeling have shown promising results in improving functional recovery after TBI in animals [11–15].

Methamphetamine is a potent psychostimulant that alters the release and reuptake of dopamine, norepinephrine, and serotonin. Large doses of methamphetamine result in neuronal toxicity and the induction of cell death throughout the brain [16]. However, low dose methamphetamine may be protective after oxygen glucose deprivation by decreasing excitatory synaptic potential, and prevents cell death by modulating cytokines and neurotrophic factors [17,18]. Recently, in a lateral fluid percussion injury model of rat, low dose methamphetamine treatment at three hours after TBI significantly increased the presence of immature neurons, and reduced apoptotic cell death, as well as improved both behavioral and cognitive dysfunctions [15].

Prior methamphetamine studies focused on histological investigation [15,17,18]. The therapeutic effects of low dose methamphetamine treatment of TBI can be dynamically morni-

tored and longitudinally investigated using magnetic resonance imaging (MRI) [19,20]. MRI, including T2-weighted imaging (T2WI), susceptibility weighted imaging (SWI), diffusion tensor imaging (DTI) and cerebral blood flow (CBF) measurement with the arterial spin labeling (ASL) technique, provides a preferable way to track biostructural damage and changes of the brain after TBI [19,20]. DTI permits assessment of diffuse axonal injury after brain injury [21–23]. Fractional anisotropy (FA) deduced from DTI is reduced in major white matter tracts in the central areas of the injured brain [21,22], and is increased in the recovery regions surrounding the impact core after cell-based treatment of TBI in rats. This increase in FA has been attributed to axonal remodeling and increased oligodendrocyte generation [19]. Thus in the present study, we employed MRI to assess the effects of low dose methamphetamine treatment on neuronal recovery following a controlled cortical impact (CCI) model of TBI in rats.

Materials and Methods

All experimental procedures were conducted and performed in accordance with guidelines for animal research under a protocol approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital (No.1051). MRI scan and data analysis, TBI surgery and methamphetamine treatment, neurobehavioral functional tests and histological measurements, were performed in a double-blind fashion. Three rats died within the 6 week test period and before completion of all MRI scans and were excluded from measurement and analysis.

TBI Model

Young male Wistar rats (Charles River, Wilmington, MA) weighing 200–300 g at arrival were kept at the animal facility for 7 days before initiating the experiments. Rats were anesthetized with chloral hydrate (350 mg/kg). Body temperature was maintained at 37°C with a feedback-regulated water recirculating pad. Blood gases and blood pressure were monitored during the surgical procedure by placing PE-50 catheters into the femoral artery and vein to obtain blood. The head of each rat was then mounted in a stereotaxic frame in a prone position and secured by ear bars and an incisor bar. Two 10 mm-diameter craniotomies were performed adjacent to the central suture, midway between the lambda and the bregma, leaving the dura mater over the cortex intact. The left craniotomy confined the location of experimental impact while the right one allowed for the lateral movement of cortical tissue. Injury was induced by a pneumatic impact device [24] on the intact dura. A single strike was delivered at 4 m/sec with a 2.5 mm of compression to the left cortex with a pneumatic piston containing a 6 mm-diameter tip. After the impact, the bone plate was not replaced and was sealed with bone wax, the skin was then sutured with 4-0 surgical thread. Buprenex (0.05 mg/kg) was subcutaneously administered to animals after brain injury for analgesia. This animal model is a clinically relevant model of TBI, which produces a severe TBI with contusion lesion affecting part of the motor and primary somatosensory cortex, and has been used for many years [25]. Animals were provided with 10 mL warmed saline via subcutaneous injection immediately after surgery and monitored 3–4 times daily within the first 48 hours following the insult and had free access to food and water.

Methamphetamine Treatment

Eight hours after the TBI, rats were anesthetized by isofluorane inhalation. An incision was made at the inguinal crease to expose the femoral vein. An Alzet osmotic pump (DURECT Corp, Cupertino, CA) connected to a catheter was placed within the crease with the catheter extending from the pump and placed into the femoral vein. The incision was closed with 4-0 vicryl sutures. One group of rats, referred to as the treated group (n = 10), were treated with a bolus dose of 0.42 mg/kg methamphetamine via the saphenous vein followed by continuous infusion intravenously (iv) with 0.05 mg/kg/hr for 24 hrs. Animals, assigned as the control group (n = 10), received the same volume of saline as bolus iv dose followed by continuous iv infusion with 6.6μL/hr for 24 hrs. At 48 hours after pump placement, rats were again briefly anesthetized with isoflurane, the pump and femoral vein catheter were removed, and the incision in the inguinal area was sutured with 4-0 absorbable suture and sealed with skin glue.

MRI Measurements

MRI measurements were performed with ClinScan 7T system, which combines Bruker-Biospin hardware (Bruker-Biospin, Ettlingen, Germany) interfaced to Siemens software, Syngo (Siemens, Erlanger, Germany). A birdcage type coil was used as transmitter and a quardrature half-volume coil as receiver. MRI scans were performed one day before TBI, 1 and 3 days post TBI and then weekly for 6 weeks. Pulse sequences included T2WI, SWI, and DTI. A fast gradient echo imaging sequence was used for reproducible positioning of the animal in the magnet at each MRI session. Stereotactic ear bars were used to minimize movement during the imaging procedure. During MRI measurements, anesthesia were maintained using medical air (1.0 L/min) and isoflurane (1.0–1.5%). Rectal temperature was maintained at 37°C using a feedback controlled water bath.

T2WI was acquired using a Carr-Purcell-Meiboom-Gill multislice and multiecho (six echoes) sequence. A series of six sets of images (13 slices for each set) were obtained using echo times (TEs) of 15, 30, 45, 60, 75, and 90 ms and a repetition time (TR) of 4.5 sec. Images were produced using a 32×32 mm^2 field-of-view (FOV), 1 mm slice thickness, 128×128 image matrix. T_2 map was obtained by exponentially fitting the six images pixel-by-pixel with different echo times for each slice. SWI employed a specialized 3-dimensional gradient echo sequence with TE = 10 ms, TR = 40 ms, flip angle of 15°, $32\times32\times24$ mm^3 FOV, $256\times192\times64$ matrix (a resolution of $125\mu m\times167\mu m\times375\mu m$), and flow compensation in all three directions. DTI was acquired using a spin-echo sequence with pulsed diffusion weighted gradients and echo-planar readout. The field of view was 32×32 mm^2; 128×128 imaging matrix, 1 mm slice thickness with 13 slices, TR = 10 s and TE = 50 ms, 1 shot, 2 averages, 1 DTI baseline of b = 0 s/mm^2, 20 directions of diffusion gradients with b = 900 and 1800 s/mm^2, respectively, for each slice.

Ex vivo MRI Measurements

Q-ball based DTI [26] was performed using a pulsed gradient spin echo sequence with the following parameters: 128×128 matrix; 32×32 mm^2 FOV; 1 mm slice thickness with 13 slices; TR = 1.5 s; TE = 38 ms; δ = 10 ms; Δ = 18 ms; b = 900 s/mm^2 at 128 directions and one baseline with b = 0; four signal average; total acquisition time was approximate 27 hours for Q-ball imaging.

Neurobehavioral Functional Tests

Neurological function was monitored by modified neurological severity scores (mNSS) [27] and foot-fault test [28]. Baseline neurological function was established for all animals prior to TBI and again on days 1, 7, 14, 21, 28, 35, and 42 post brain injury. On day 38 to day 42 following TBI, the Morris Water Maze (MWM) test was performed to assess the impact of methamphetamine on cognitive function (learning and memory) [29].

Immunohistochemical Staining

Animals were euthanized after completing the last MRI measurements and functional tests at 42 days post TBI. Rats were anesthetized with intraperitoneal injection with chloral hydrate, and perfused transcardially with saline, followed by 4% paraformaldehyde. Brains were isolated, post-fixed in 4% paraformaldehyde for 2 days at room temperature, and then processed for paraffin sectioning. Using light microscopy and a laser scanning confocal microscope (Zeiss LSM 510), we measured a composite index of independent measurements from multiple immunohistochemical stainings for axons (Bielschowsky's silver and Luxol fast blue, BLFB [30]; monoclonal antibody to non-phosphorylated neurofilaments, SMI-32 [31]), dendrites (microtubule-associated protein 2, MAP-2 [32]) and synapses (synaptophysin, SYP [33]). Data were expressed as density, or percentage of area of immunoreactive proteins, to the area of the FOV. Lesion areas were measured as a percentage to the ipsilateral hemisphere areas with hematoxylin and eosin, H&E [34], immunohistochemical stained slices.

MRI Data and Statistical Analysis

MRI image analysis was generally performed with homemade software, Eigentool [35]. Q-ball DTI analysis was performed using Camino software [36].

The TBI lesion was identified on the MRI T_2 map. The ipsilateral lesion area on each slice of the T_2 map was specified by those pixels with a T_2 value higher than the mean plus the twice standard deviations provided by the normal tissue of the same animal on the pre-TBI T_2 map. Lesion volume was obtained by adding all the areas measured on individual slices and multiplying by the slice thickness. The volume of the lateral ventricle was measured on T_2 maps at a fixed structural location presented by four contiguous coronal T_2 slices using the same criterion as described above to identify the ventricular area on each slice. The ventricular volume was obtained by adding all the areas measured on individual slices and multiplying by the slice thickness.

Two MRI regions of interest (ROI) were identified as the TBI core and recovery areas for analysis of MRI parameters. The first ROI, referred to as, the TBI core, was demarcated on the T_2 map obtained 6 weeks after TBI, by using the T_2 value threshold of mean plus two standard deviations based on the T_2 value measured in the pre-TBI T_2 map for each animal [19]. The second ROI, referred as the, TBI recovery area [19], was demarcated by subtracting the TBI core from the TBI lesion area in T_2 maps obtained 24 hrs after TBI.

SWI was employed to identify hemorrhagic lesions after TBI and to demarcate the TBI lesion from the ventricle in rat brain [20].

The FA map from DTI was first warped to the corresponding T_2 map; then, the TBI core and recovery ROIs obtained from the same slice of T_2 map were, respectively, loaded onto the FA map for measurement. The present FA data were normalized to pre-TBI FA values.

Diffusion standard deviation (SD) map [37], derived from Q-ball imaging, was created based on calculating the deviation of diffusivity from a sphere for each voxel in the image. If diffusion is constrained by tubular structures, the SD will possess a non-zero value based on the complexity of the structure [37].

A mixed model, analysis of variance (ANOVA) and covariance (ANCOVA) was performed. The analysis started testing the treatment group and time (without baseline time point) interaction, followed by testing the group difference at each time point, or the time effect for each treatment group, if the interaction or the overall group/time effect was detected at the 0.05 level. Subgroup analysis was followed to test the difference at each group and time level using the paired two-tailed *t*-test. Two-sample *t*-test was performed to check the balance of the data at the baseline, as well as to test the treatment effect at day 42 post TBI for histological evaluation. Pearson's correlation analysis was applied between MRI values obtained at 6 weeks after TBI and histological measurements. MRI and histological measurements are summarized as mean and standard error (SE).

Results

The lesion volumes of TBI damaged cerebral tissue were demarcated by elevated values in T_2 maps and measured at 24 hrs, 72 hrs and weekly from 1 to 6 weeks post TBI in rats. T_2 maps of representative rats are shown in figure 1, which depict the evolution of T_2 values for a methamphetamine treated rat (the 1st row) and a saline treated rat (the 2nd row), respectively. Lesion volumes at 24 hrs after TBI, 65.03 ± 8.54 mm^3 for the treated rats and 56.71 ± 5.15 mm^3 for the control rats, exhibited no initial statistical difference ($p>0.41$) between the two groups. At 6 weeks after TBI, lesion volumes were 25.51 ± 12.14 mm^3 for the treated, and 32.49 ± 9.45 mm^3 for the control, respectively, and no statistical differences ($p>0.65$) were detected. Methamphetamine treatment did not significantly reduce lesion volumes compared to the controls during 6 weeks after TBI in rats, and the lesion volumes are quantitated in Fig. 2 a.

The two lower rows of T_2 maps in figure 1, from a methamphetamine treated rat (the 3rd row) and a saline treated rat (the 4th row), respectively, exhibited the expansive profiles of cerebral ventricles after TBI. Pre-TBI, the lateral ventricular volumes were 3.12 ± 0.09 mm^3 in each hemisphere and were demarcated using T_2 maps. All animals exhibited similar ventricular volumes on both hemispheres, and no differences were detected between the two treatment groups. After TBI, ventricular volumes were measured at 24 hrs, 72 hrs, and then weekly from 1 to 6 weeks. No benefits of low dose methamphetamine treatment were observed on reducing ventricular expansion in the treated rats, compared to the controls (Fig. 2 b). At 6 weeks after TBI, the ipsilateral ventricular volumes were measured and found to be 7.93 ± 1.29 mm^3 for the treated rats, and 10.71 ± 3.32 mm^3 for the control animals. Correspondingly, in the contralateral side, the ventricular values were 4.55 ± 1.00 mm^3 for the treated and 5.29 ± 1.20 mm^3 for the control groups. No significant differences were detected between the two groups ($p>0.45$ for ipsilateral side, $p>0.64$ for the other side).

The temporal profiles of FA values, normalized to pre-TBI measurements, of the TBI core and recovery ROIs were calculated (Fig. 2 c) and used for analysis. The FA values obtained from the TBI core ROIs remained low within six weeks after TBI for both control and treated groups, and no significant differences were detected in FA measurements of the core regions between the two groups. However, the FA values in the TBI recovery ROIs increased during 6 weeks post TBI in both control and treated groups. The methamphetamine treated group revealed larger increases than the control group. Significant increases of FA values obtained from the TBI recovery ROIs were detected between the treated and control groups starting at 5 weeks after TBI. The values of the normalized FA for treated and control groups were 1.13 ± 0.21 versus $0.94\pm.09$ at 5 w ($p<0.03$), and 1.21 ± 0.14 versus 1.03 ± 0.15 at 6 w ($p<0.02$), respectively, after TBI.

T_2 values of cerebral tissue were measured in the TBI core and recovery ROIs for all animals in the study (Fig. 2 d). There were no significant differences between the methamphetamine and saline treated animal groups for either TBI core or recovery

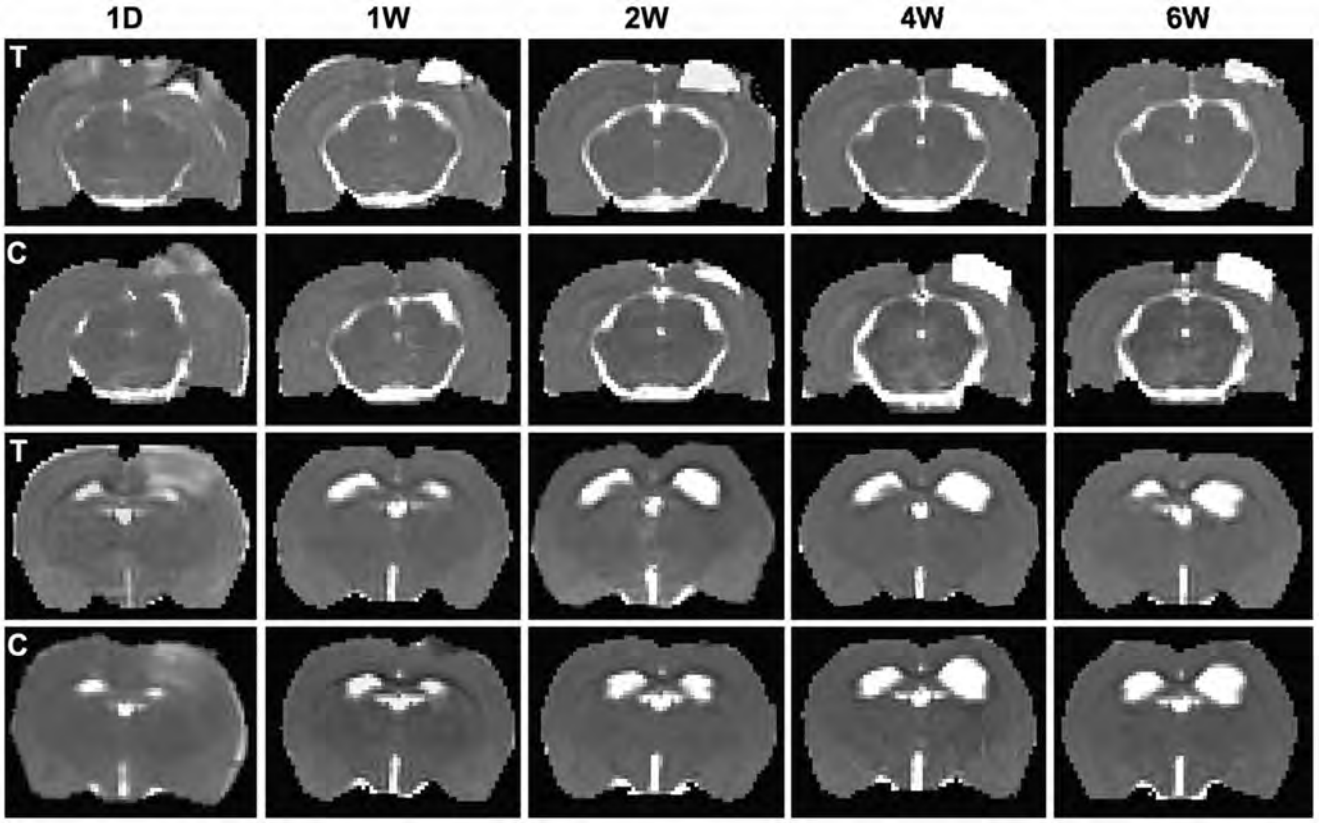

Figure 1. T_2 maps demonstrating the evolution of TBI lesion volumes (two upper rows) from representative low dose methamphetamine treated (T) and saline treated (C) TBI rats. The two lower rows are also T_2 maps, which demonstrate the evolution of ventricular expansion for treated (T) and control (C) rats. Although the T_2 maps obtained at 6 weeks after TBI showed smaller lesion and ventricular volumes in the treated rats (the 1st and 3rd rows) compared with the control rats (the 2nd and 4th rows), no statistically significant differences were found between the treated ($n = 10$) and control ($n = 10$) groups during the 6 weeks after TBI.
doi:10.1371/journal.pone.0061241.g001

regions, respectively. The temporal changes of T_2 values of injured cerebral tissue, however, demonstrated a significant time effect after TBI for both groups. Starting at 14 days post TBI, T_2 values of cerebral tissue were significantly different between the TBI core and recovery regions for both animal groups throughout the 6 week measurement time ($p<0.05$).

For the immunohistochemical measurements, significant increases in MAP-2 (Fig. 3 a, $p<0.02$) and SMI-32 (Fig. 3 b, $p<0.02$) were observed in the ipsilateral TBI lesion boundary in the treated rats with the values of $12.58\pm2.12\%$ (MAP-2) and $9.09\pm2.25\%$ (SMI-32), compared to the control rats with the values of $9.98\pm1.68\%$ (MAP-2) and $7.36\pm1.52\%$ (SMI-32), respectively. Moreover, BLFB stained axons and myelin, respectively, (Fig. 3 c) were histologically measured in the TBI boundary ROI for changes in cerebral white matter. A significant increase ($p<0.001$) was detected in the treated group, $25.94\pm1.41\%$, compared with control group, $17.05\pm2.95\%$. However, no significant differences were detected in synaptophysin expression between the treated, $17.27\pm5.44\%$, and the control rats, $17.82\pm5.44\%$ (Fig. 3 d). Analysis of H&E sections, showed percent lesion volumes of $10.4\pm4.8\%$ for the treated rats and $14.3\pm5.2\%$ for the control, and no differences were detected between the two groups ($p = 0.10$).

A significant Pearson's correlation was detected between normalized FA values obtained at 6 weeks after TBI and measurements of BLBF staining in TBI boundary ROI ($R = 0.54$, $p<0.02$).

Reduced functional impairments were detected in the low dose methamphetamine treated group as compared with the control group. Significant improvements by methamphetamine treatment were detected in mNSS from 2 to 6 weeks ($p<0.05$), as shown in Fig. 4 a; and in foot-fault tests from 3 days to 6 weeks after TBI ($p<0.05$), as shown in Fig. 4 c for the hindlimb and Fig. 4 d for the forelimb. However, no significant improvement was detected with Morris Water Maze test, see Fig. 4 b ($p = 0.12$).

Figure 5 shows ex-vivo MRI from a representative control rat sacrificed at 6 weeks after TBI. The fiber orientation map, Fig. 5 a, was derived from a Q-ball MRI scan. The area within the blue frame is enlarged (Fig. 5 b), and indicates well-oriented fibers (few crossing patterns) in cortex (blue and yellow arrow heads) surrounding the TBI lesion core, that is referred to as the T_2 map (cyan arrow in Fig. 5 e). With elevated values, both SD (Fig. 5 c) and FA (Fig. 5 d) maps identified the white matter fiber tracts along two sides of the lesion core (blue and yellow arrow heads in Fig. 5 c & d), and the FA map exhibited a higher image contrast than the SD map. The areas with reduced differences between FA and SD maps may reflect the reorganization of axonal fibers taking place after TBI, and the well organized fibers were oriented in a single direction. These MRI outcomes were consistent with the histological results obtained with BLFB staining (blue and yellow arrow heads in Fig. 5 h, enlarged from Fig5. g & f).

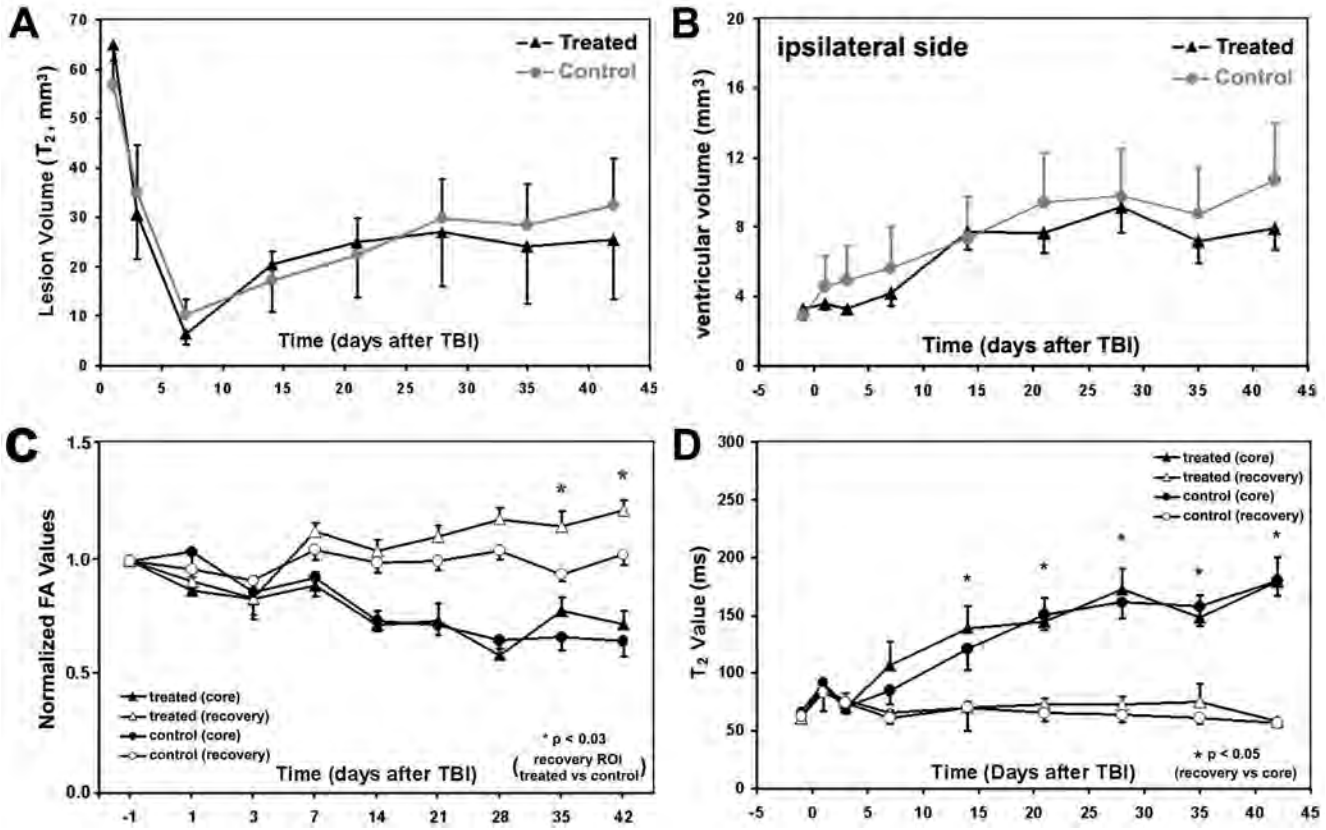

Figure 2. Lesion (A) and ipsilateral ventricular (B) volumes measured on T_2 maps after TBI were charted for both treated ($n=10$) and control ($n=10$) rats. Although the lesion and ventricular volumes were smaller in the treated rats, i.e. evident at 4 weeks for lesion and 3 weeks for ventricle after TBI, than that in the control rats, no statistically significant differences were found between the two groups during the 6 weeks after TBI. Normalized FA values (C) of recovery area demonstrated significant differences between the groups starting 3 weeks after TBI in rats. T_2 values of both core and recovery regions (D) did not show statistical differences between the two groups in 6 weeks after TBI; however, significant differences were observed between core and recovery regions starting 2 weeks after TBI for all rats.
doi:10.1371/journal.pone.0061241.g002

Discussion and Conclusions

This study demonstrated that low dose methamphetamine treatment starting at 8 hours after traumatic insult in rats significantly improved the recovery of behavioral functions in rats by lowering mNSS and foot-fault scores after TBI, compared with saline treatment. The treatment promoted neuronal recovery after TBI, which was detected by MRI measurement of FA and confirmed by immunohistochemical outcomes. The mean relative FA values of the TBI recovery area exhibited significant increases in the treated group at 5 and 6 weeks after TBI, compared with the control group ($p<0.03$). The increased FA, indicating a more reorganized white matter, may contribute to the functional recovery after TBI in rats.

FA values have large variations dependent on the location of the ROI in rat brain. Using the normalized FA, referred to as, pre-TBI FA values, of the same ROI and the same animal, may provide a more precise measure of white matter changes after TBI in rat with or without treatments, since each ROI location differed among the animals.

Based on the evolution of T_2 values measured from all animals in the study, it was reasonable to demarcate TBI damaged cerebral tissue into core and recovery tissue by using T_2 maps obtained at 24 hours and 6 weeks after TBI. Fig. 2 d indicates that T_2 values of cerebral tissue in core ROI, independent of treatment after TBI, increased from 1 week after TBI and then retained elevated values, while T_2 values of cerebral tissue in recovery ROI decreased during the first week after TBI and remained the same for 6 weeks. T_2 values were very similar and no differences were observed between the treated and control groups for either core or recovery ROIs, respectively. Meanwhile, T_2 values of cerebral tissue exhibited significant differences ($p<0.05$) between the core and recovery ROIs starting at 2 weeks after TBI for both groups. Furthermore, T_2 values of cerebral tissue from the core ROI approached the T_2 values of cerebral spinal fluid, and T_2 values from recovery ROI tended towards pre-TBI values. These results indicated that cerebral tissue in the core ROI may be irreversibly damaged after TBI, independent of methamphetamine or saline treatments, which suggest that effective treatment of TBI may be attributed to improved biophysical functions of cerebral tissue in the recovery region.

MRI measurements of T_2 maps did not detect the therapeutic effects of the low dose methamphetamine treatment on reducing lesion volume (core size) and ventricular expansion up to 6 weeks after TBI in rat, although the mean values of lesion volume and expansion rate of ventricular volume were decreased ($p = 0.20$ for lesion volume and $p = 0.32$ for ventricular volume) in the treated group as compared to the control group (Fig. 2 a & b). This result was consistent with histological H&E measurements of TBI lesion volumes. The neuroprotective effect of the low dose methamphetamine treatment of stroke is dose and time dependent [18]. A prior study indicates that immediate treament of embolic stroke in rats

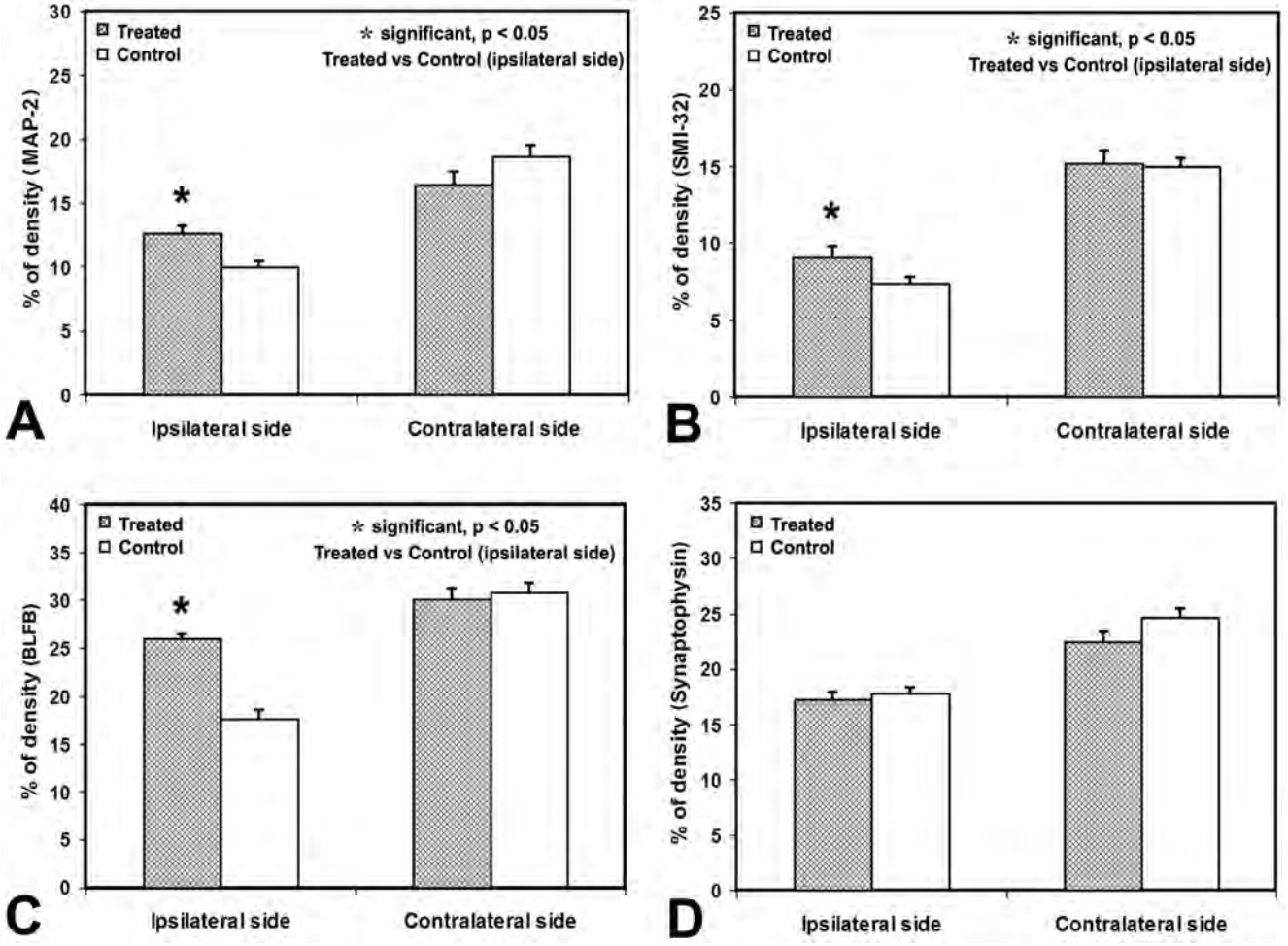

Figure 3. Measurements of densities of MAP-2 staining (A), SMI-32 staining (B), and BLFB staining (C), demonstrated that low dose methamphetamine treatment of TBI significantly increases neurons density and axonal reorganization in the ipsilateral hemisphere in rats. However, no differences were found for synaptophysin staining (D). No differences were found with immunohistochemical density stainings in the contralateral hemisphere of rat brain after TBI.
doi:10.1371/journal.pone.0061241.g003

with methamphetamine for 24 hours at a dose of 0.1 mg/kg/hr, or treatment initiated at 12 hours with a dose of 1.0 mg/kg/hr did not reduce infarct volume [18]. Thus, the curent results in TBI are consistent with the previous results, that no statistical neuroprotective effects were observed in the delayed methamphetamine treatment in stroke. Expansion of the ipsilateral ventricular volume after brain injury may indicate the loss of cerebral tissue, and ventricular volume is a sensitive MRI index of brain atrophy after stroke in rats [38,39]. In previous experimental studies of embolic stroke, therapeutic effects of delayed Erythropoietin treatment in adult rats or Sildenafil treatment in aged rats were detected starting at 4 weeks or 6 weeks after stroke by the reduced expansion rate of the ipsilateral ventricular volume demarcated using MRI T_2 maps, respectively [38,39]. For TBI rats, experimental studies demonstrated a significant increase of lateral ventricular volume at one year [40], and a significantly reduced increase of lateral ventricular volume starting at 3 weeks post TBI in bone marrow stromal cell treated rats, compared to saline treated rats [20]. The results of the present study, thus, suggest that methamphetamine treatment, initiated at 8 hours after TBI with the selected dose (0.05 mg/kg/hr), does not reduce brain atrophy within 6 weeks post traumatic insult.

The methamphetamine treatment of TBI rats in the present study does not have significant effects on neuroprotection, but enhances neurorestoration (neuronal and functional improvement), similar to the delayed pharmacological treatments of stroke in rats [38,39]. As in stroke studies [23,37–39], the delayed treatments of TBI promoted neurorestorative process in cortical tissue, such as axonal density and reorganization, which fosters improved behavioral function. Without reducing lesion volume, cell-based treatments of stroke in rat also improved functional recovery from the short term (4 weeks) to the long term (1 year) [41,42]. Therefore, the delayed low dose methamphetamine treatment of TBI in rats may improve functional outcome by enhancing biophysical functions of cerebral tissue in the recovery region, although the treatment did not reduce lesion volume and brain atrophy within 6 weeks post TBI.

Extensive axonal degeneration is the common result of traumatic brain injury [4,6]. Injury, however, inversely induces axonal sprouting [43,44], and new synaptic connections and myelination are present in both injured and neighboring regions [45]. White matter is reorganized, especially in the areas of the corpus callosum. Proper treatment of TBI may enhance brain reparative processes [19,20], similar to stroke [23,38,39]. MRI FA

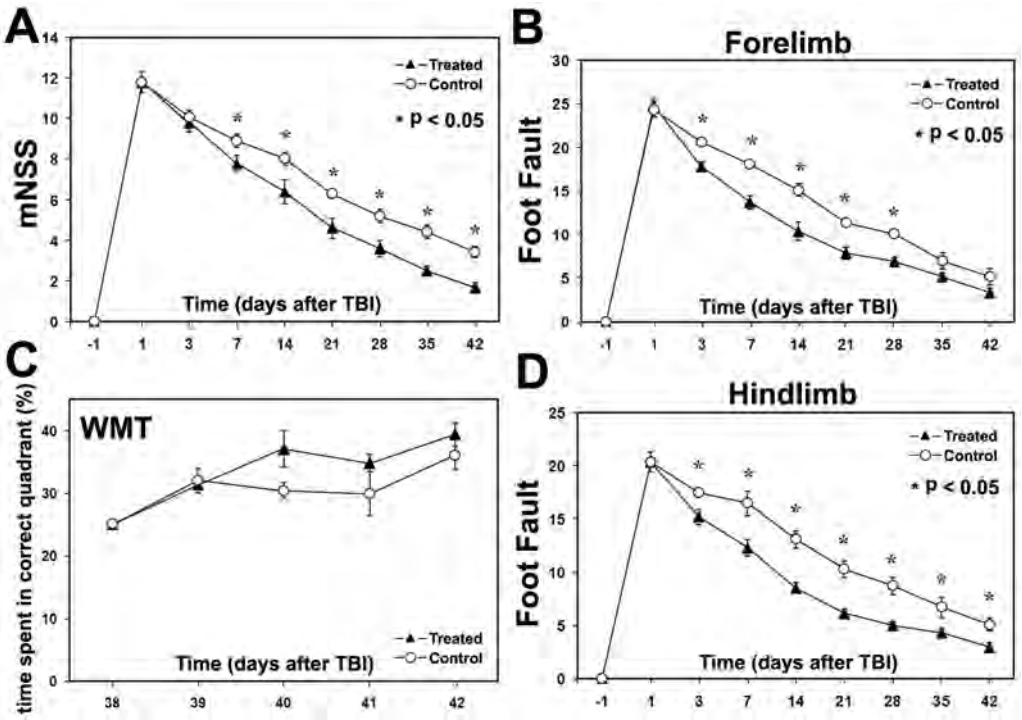

Figure 4. Functional tests demonstrated that low dose methamphetamine treatment significantly lowered mNSS (A) and reduced foot faults (C: forelimb; D: hindlimb) in treated rats starting at 1 week after TBI. However, no differences were observed in Morris water maze test (B) between the treated and control rats.
doi:10.1371/journal.pone.0061241.g004

was able to monitor well-reorganized white matter recovery, and similar results have been described for the detection of white matter recovery after treatment of stroke in rats [19,23]. In the present study, our data demonstrated a remarkable increase in FA of the white matter extending from the corpus callosum to the boundary of the TBI lesion in the treated animals, in contrast to the controls (Fig. 2 c), suggesting that low dose methamphetamine treatment facilitates white matter reorganization involving the corpus callosum.

FA may be unable to detect white matter reorganization when the white matter fiber bundles cross, and FA merely expresses an overall lower value because of the assumption of a Gaussian diffusion inherent to the tensor model [46–48]. Solving the orientation distribution function (ODF), which is used to describe the directionality of multimodal diffusion in regions with complex fiber architecture, permits more accurate detection of crossing fibers [26,36,49]. This calculation involves a complex set of Q-space DTI analysis, including diffusion spectrum imaging (DSI)

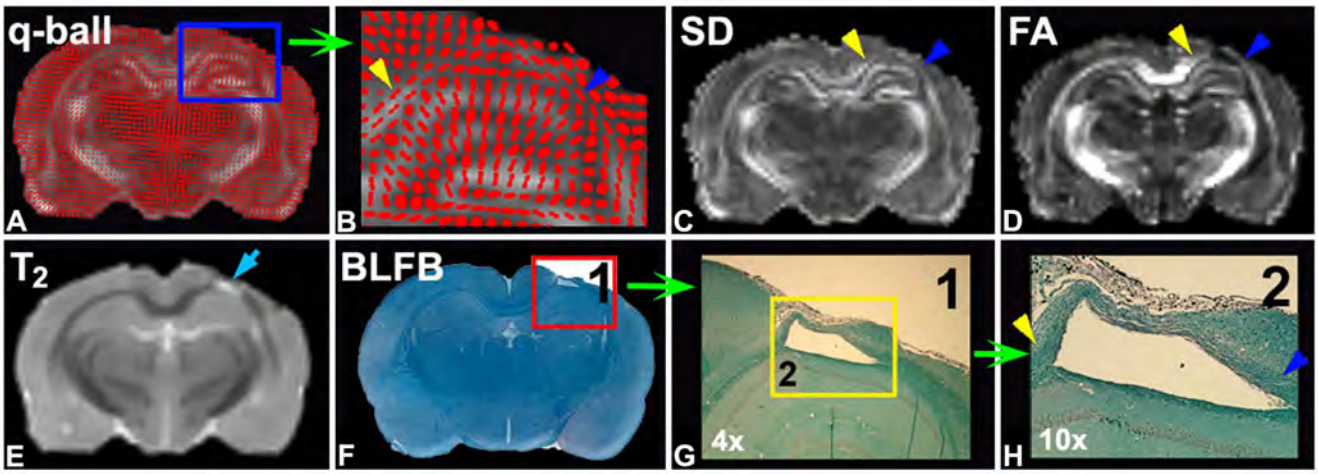

Figure 5. An ex vivo MRI scan of a control rat q-ball map (A), which is enlarged (B) to view fiber crossing, showed few crossing fibers (blue and yellow arrow heads) surrounding the TBI lesion core (E, cyan arrow). Both SD (C) and FA (D) maps detected the reorganized white matter (blue and yellow arrow heads), which was consistent with BLFB staining (blue and yellow arrow heads in H; G: 4× enlarged, red frame in F; H: 10× enlarged, yellow frame in G).
doi:10.1371/journal.pone.0061241.g005

[49], Q-ball [26] and persistent angular structure MRI (PASMRI) [36]. In addition, diffusional kurtosis imaging (DKI) has been introduced as a quantitative measure of the degree to which the diffusion displacement probability distribution deviates from a Gaussian form [50]. Indeed, DKI has been shown to provide the information necessary to solve the ODF [51], whereas the apparent kurtosis coefficient (AKC) can provide additional information on neural tissue micro-architecture [52], such as cellular compartments and membranes, and AKC is more sensitive to axonal density than orientation. Generally, DKI acquires images with multiple b-value shells but with less direction than Q-ball does, thus DKI provides favorable quantitative diffusion probability distribution, but Q-ball with a higher resolution angular distribution. Therefore, the advantage of the SD map is that it can be concomitantly obtained with a fiber crossing map using a single measurement of Q-ball, while angular resolution of AKC is generally too low to simultaneously produce a high quality fiber crossing map. Both SD and AKC are sensitive to early stages of white matter reorganization, and are superior to FA in the detection of white matter reorganization with prominent crossing axons [19,37]. However, solving the ODF requires high angular resolution MRI data. The more directions the MRI scan contains, the more accurate the ODF produced, and the more time the MRI scan takes. Thus, very high angular resolution DKI and Q-ball DTI are rarely employed for in vivo studies.

SD and FA should show a similar pattern if the white matter bundle is well organized along a single direction [19]. The BLFB stained slices and MRI fiber crossing maps of the current study (Fig. 5) showed that the majority of axonal bundles were well organized into a single direction and are present only in small areas with crossing bundles. These results indicated that fiber crossing, at least 6 weeks after TBI, has a minor affect on FA values of TBI recovery cerebral tissue. Thus, the lower FA values in control rats than in the methamphetamine treated rats were primarily caused by fiber density rather than by fiber crossing, which was also alternatively confirmed by the significant Pearson's correlation ($R = 0.54$, $p<0.02$) between the FA measured 6 weeks after TBI and histological BLFB measurements. The temporal profiles of FA measurements (Fig. 2 c), suggested that low dose methamphetamine treatment after TBI improves white matter reorganization from 5 weeks after brain traumatic insult in rat, compared with saline treatment.

Immunocytochemically, SMI 32 visualizes neuronal cell bodies, dendrites and some thick axons, especially for neocortical pyramidal neurons [53]. The significant increase of SMI-32 and MAP-2 immunoreactive proteins, thus, suggests that methamphetamine treatment after TBI promotes the development of dendrites. Meanwhile, the treatment also increased axonal myelination, which was indicated by a significant increase of BLFB immunoreactive axons in the ipsilateral hemisphere of TBI rats. Therefore, these results indicated that methamphetamine treatment improves neuronal recovery in TBI rats. However, the synaptophysin data did not show any significant differences between the treated and control rats, and the reason for this is unclear.

Neuronal fiber reorganization after injury may play an important role in fuctional recovery [41,42]. We found that 12 hour post stroke methamphetamine treatment at an infusion dose of 1.0 mg/kg/hr for 24 hours in rats did not reduce infarct volume examined at 7 days post injury, but, did improve neurological scores in rats compared with saline treated controls [18]. In the present study, our data demonstrated that low dose methamphetamine treatment of TBI reduces neither TBI lesion volume, nor ventricular volume expansion, but, the treatment increased white matter reorgnization, and hence, functional outcomes over a 6 week period after the TBI insult, compared to the control. This result indicated that, as in the stroke model, delayed administration of very low dose methamphetamine has a minor effect on neuroprotection, but yet still improves functional outcome in TBI rats; which implies that reorganization of white matter may be a major contributor to the functional recovery after brain injury. The improved functional outcome with low-dose methamphetamine treatment also suggests that a significant increase in cortical neuron activity in methamphetamine treated rats may enhance sensorimotor functions, such as mNSS and foot-fault, without improving cognitive function, as measured with the Morris Water Maze test.

Using a different treatment protocol (0.845 mg/kg in bolus injection, followed by 0.5 mg/kg/hr×24 hrs infusion), timing (3 hrs after TBI) and traumatic model [15], methamphetamine treatment of TBI in rat was recently reported to improve not only behavioral function in mNSS and foot fault tests, but also cognitive function in the Morris Water Maze test 27 days after TBI insult. Thus, combined with our present results, these data suggest that the functional recovery after TBI in rats is dependent on dose and time of methamphetamine treatment.

Methamphetamine is a potent psychostimulant that alters the release and reuptake of dopamine, norepinephrine, and serotonin [18]. In a transient middle cerebral artery occlusion model in the rat, low dose of methamphetamine was found to mediate neuroprotection through activation of a dopamine-PI3K-AKT signaling pathway [18]. Similar results were found in a rat lateral fluid percussion injury TBI model [15]. In the current study, the mean TBI lesion volume in the treated group (25.51 ± 12.14 mm^3) was smaller than that in control group (32.49 ± 9.45 mm^3) although it did not reach statistical significance at 6 weeks after TBI. We note, that the dose used in the present study was one tenth of that used in the earlier studies, and therefore a higher dose may produce a more robust effect [15]. Therefore, the very low dose methamphetamine treatment of rat initiated at 8 hours post TBI may provide a minor neuroprotective effect. Several previous studies have demonstrated increased axonal density detected by MRI FA and corresponding BLFB staining [19,23,37–39], and cell-based treatment 6 hours after TBI demonstrated neurorestorative effects compared with untreated animals [54]. Thus, based on the MRI FA detected increase of axonal bundles and confirmed with corresponding BLFB staining data, the low dose methamphetamine treatment may promote neurorestorative effects, since it induces white matter reorganization and functional recovery after TBI. However, the exact mechanisms underlying the therapeutic effects of low-dose methamphetamine for TBI are not clear, and merit further investigation.

In summary, very low dose (a bolus injection of 0.42 mg/kg followed by an infusion of 0.05 mg/kg/hr for 24 hrs) methamphetamine treatment starting at 8 hours after TBI in rats did not significantly reduce lesion volume and brain atrophy, however, it did affect white matter structure and remodeling, and neurological function at 6 weeks after traumatic insult. In addition, MRI provided sensitive and insightful indices underlying this functional benefit.

Author Contributions

Conceived and designed the experiments: QJ MC DJP AM. Performed the experiments: GLD CQ HW. Analyzed the data: LL QL JSB. Contributed reagents/materials/analysis tools: SPN. Wrote the paper: GLD QJ DJP MC.

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